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13. SUPPLEMENTARY NOTES

14. ABSTRACT Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, but the underlying mechanisms are unknown. Many ASD associated mutations modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) to produce bowel disorders. NL3 mice express a neuroligin-3 mutation identified in ASD patients and are more responsive to the GABA neurotransmission in the brain. This work aims to study the spatiotemporal distribution patterns of NL3 in gut tissue from these mice in order to determine biological mechanisms contributing to GI dysfunction in patients with ASD. We will also use intracellular recording techniques to determine which cell types are responsive to GABA in the myenteric plexus and if responses differ in WT and NL3 mutant colon. We have localized neuroligin 3 protein to a subset of neurons in the mouse colon and jejunal myenteric plexus. Unexpectedly, we show localization of neuroligin-3 protein to presynaptic specializations in the myenteric plexus in both the colon and the jejunum of the mouse. Importantly, we have demonstrated that S neurons in the proximal myenteric plexus of the mouse colon are depolarized in response to local application of GABA.

15. SUBJECT TERMS presynaptic localization of the neuroligin-3 protein S neurons in mouse myenteric plexus are responsive to GABA					
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Introduction

Disorders in bowel function are common in autism. Up to 80% of ASD patients exhibit gastrointestinal (GI) problems, notably chronic constipation, but the underlying mechanisms are unknown. Many mutations identified in ASD patients modify synaptic proteins and hence alter synaptic function in the brain. We propose that some of these mutations also alter the enteric nervous system (ENS) of the GI tract to produce bowel disorders. We have shown that adhesion molecules important to synaptic function in brain including neuroligin-3 and neurexins 1 and 2 are expressed in mouse myenteric plexus. NL3 mice, which express a human neuroligin-3 mutation identified in ASD patients, are more responsive to the inhibitory neurotransmitter GABA in the brain. Our initial studies found that blockers of $GABA_A$ receptors, bicuculline and gabazine, depress motor activity in NL3 mouse colon, but to a lesser extent or do not in wild type colon. This project aims to further investigate changes in gastrointestinal function in NL3 mice compared with WT littermates by examining motility in different regions of the GI tract in *in vitro* preparations from adult and developing animals. We will use intracellular recording techniques to determine which cell types are responsive to GABA in the myenteric plexus and if responses differ in WT and NL3 mutant colon. We will also study the spatiotemporal distribution patterns of NL3 and related proteins in gut tissue from these mice. This project aims to determine biological mechanisms contributing to gastrointestinal dysfunction in patients with ASD.

Body

Localizing the neuroligin 3 protein: Synapses expressing NL3

Task 2. Localize the neuroligin 3 protein: PIs Bornstein (cell classes and mutated NL3 expression, 10 months: months 15-24) **and Hill** (synapses, 10 months: months 3-12) (immunohistochemistry and confocal microscopy); 8 adult mice.
2c. Synapses expressing NL3: Tissue from 4 WT mice labelled for NL3, GABA, 5HT, synaptophysin (and non-neuronal cells if present: S-100B, kit). (10 months: months 3-12)

This work relates to **SOW Task 2c:** Synapses expressing NL3 protein.

The localisation of neuroligin 3 and protein binding partners is not well characterised in the central and peripheral nervous systems. However, we are aware of two studies using immunohistochemical techniques to localize these proteins in the CNS (Gilbert et al., 2001, Budreck and Scheiffele, 2007).

From the CNS data, we predicted that neuroligin 3 would be a component of the post-synaptic densities at enteric synapses. To test this, we used double labelling immunohistochemistry to colocalise neuroligin 3 with a marker that labels both the somata and dendrites of myenteric neurons. Approximately 40% of myenteric neurons in the mouse colon are immunoreactive for neuronal nitric oxide synthase (nNOS) and the immunoreactivity clearly fills the entire cell body and its dendrites. These neurons have been identified as interneurons and inhibitory motor neurons and are believed to play a major role in regulation of CMMCs. Thus, we used antisera against both neuroligin 3 and nNOS in this phase of the study (nNOS; neuronal Nitric Oxide Synthase; Jackson ImmunoResearch, West Grove, USA and Neuroligin 3; Cat#129113, Synaptic Systems, Germany).

We have performed fluorescence immunohistochemistry for neuroligin-3 in mouse myenteric plexus (**Figures 1, 4, 5 and 6**). Unexpectedly, these data show that neuroligin-3 is expressed predominantly in presynaptic terminals (for example, see thin arrows, **Figure 1**). Somatic labelling was also seen (**Figure 1** lower panel, horizontal arrow). Due to these surprising results we then tested for NL3 staining in guinea pig colon myenteric plexus (using funds from NHMRC allocated for other projects) for comparison (**Figure 2**).

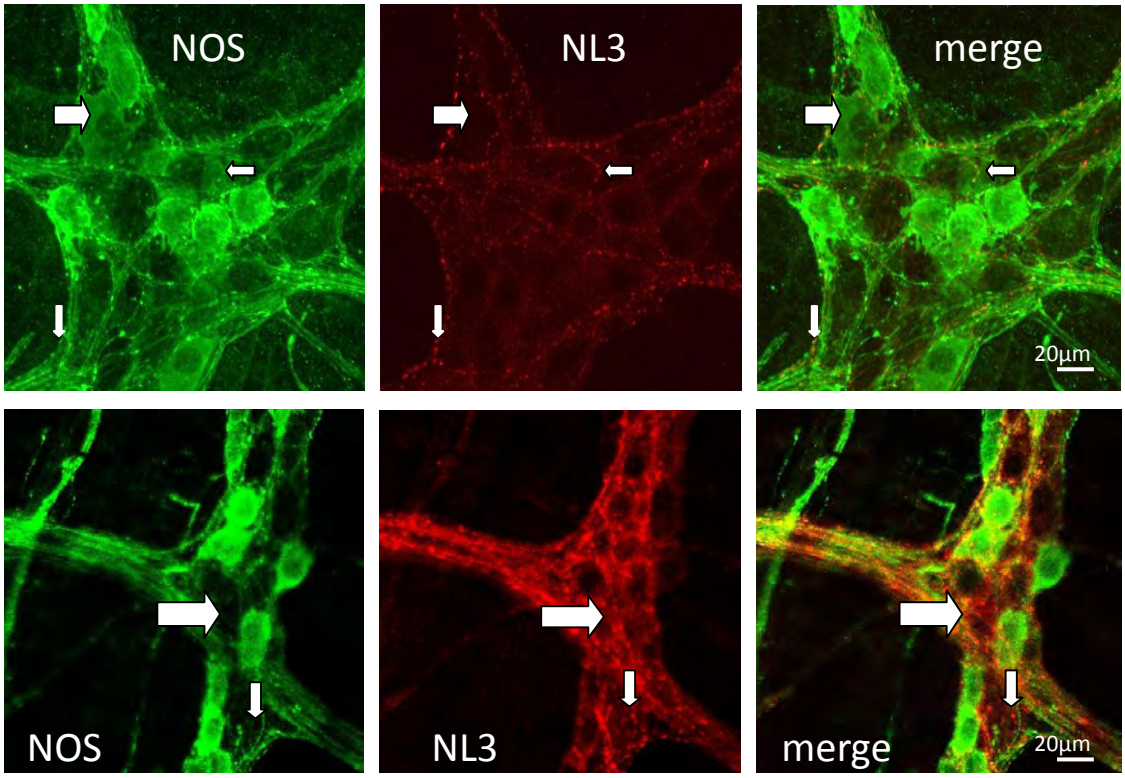

Figure 1: Localisation of nNOS and NL3 in the myenteric plexus of WT mouse colon. Representative images of immunohistochemical staining for nNOS and NL3 in WT adult mouse myenteric plexus. **Top;** thick horizontal arrow identifies a NOS positive neuron which is immunonegative for NL3**;** thin arrows identify beaded processes (putative axonal synaptic specializations) immunoreactive for both NOS and NL3. **Bottom;** thick horizontal arrow denotes a NOS negative cell body which is labelled for NL3. Vertical arrow: NL3 immunoreactive (NOS negative) presynaptic specializations. Scale bar = 20 µm.

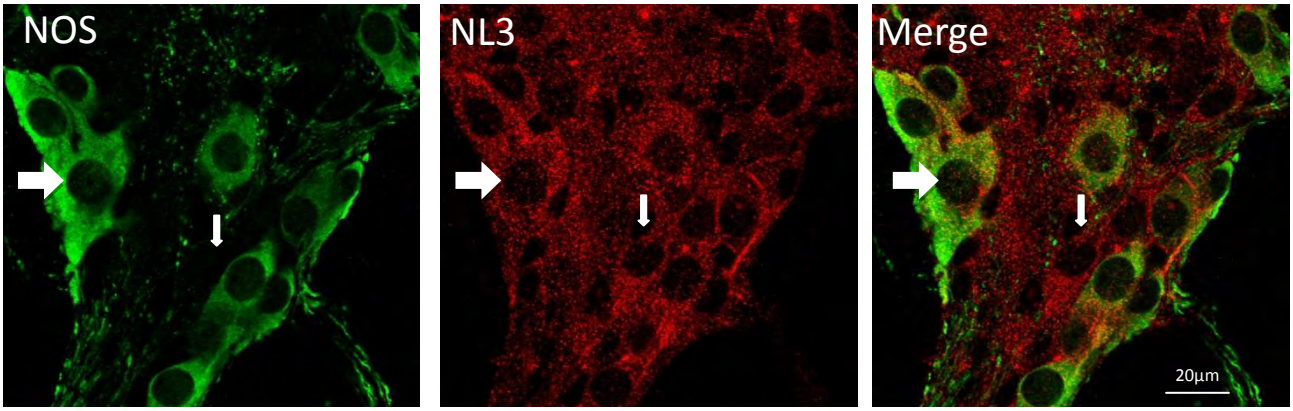

Figure 2: Localisation of nNOS and NL3 in the myenteric plexus of guinea pig colon. These images show clear neuronal localization of NL3 (red; 1:1000) and nNOS (green; 1:500) in the myenteric plexus. However, not all NL3 immunopositive neurons express nNOS. Thick horizontal arrow: neuron colocalising nNOS and NL3, vertical arrow: neuronal cell body immunonegative for nNOS but labelled for NL3.

In the guinea pig myenteric plexus, both neuronal cell bodies and varicosities showed clear labelling for neuroligin 3. Furthermore, even in the guinea-pig where both nNOS immunoreactive and unidentified neurons appear to contain neuroligin 3, no clear evidence for involvement in post-synaptic specializations was obtained. Similar results have been obtained in myenteric plexus of the jejunum of both WT and $NL3^{R451C}$ mice (**Figure 3**). Labeling in WT jejunum showed neuroligin 3 in cell bodies and varicose processes, whereas $NL3^{R451C}$ jejunum seemed to lack neuroligin 3 cell bodies (notably nNOS neurons were clearly negative for neuroligin 3) but retained significant numbers of varicose processes. Although these results remain to be quantified, they differ from studies of cultured hippocampal cells that suggest neuroligin 3 is ubiquitously expressed in neurons (Budreck and Sheiffele 2007).

A key observation from the jejunum is that there appear to be fewer neuroligin 3 labelled cell bodies in KI compared with WT (**Figure 3**). This observation will be quantified in the next 12 months of the project. Such a reduction in NL3 expression in the jejunum of mutant mice could perhaps indicate altered protein handling in the mutant as has previously been reported (Comoletti et al., 2004) however these findings need to be explored further.

In contrast to the guinea pig data, results from mouse tissue show NL3 expression in a subset of myenteric neurons. This is an important finding that needs to be quantified in the ENS, as determining the neuronal subtypes expressing NL3 will enhance our understanding of how the R451C mutation influences gastrointestinal motility in these mice. Furthermore such studies will results in a greater understanding of the role of NL3 in the wild type ENS. Given these data contrast with earlier findings in dissociated hippocampal cells (Budreck and Scheiffele, 2007); clarification of NL3 expression in the CNS may also be warranted.

The unexpected finding of a presynaptic location for neuroligin 3 suggested that the antiserum may have also localised a different protein. Accordingly, we tested the specificity using Western blot analysis and found that the antiserum recognised only a single protein band with an appropriate molecular weight (**Figure 4**). This confirms its specificity for neuroligin 3.

We have purchased antisera targeting Neurexin 1, Neurexin 2 and Neuroligin1 and are optimizing them for use in mouse enteric tissue.

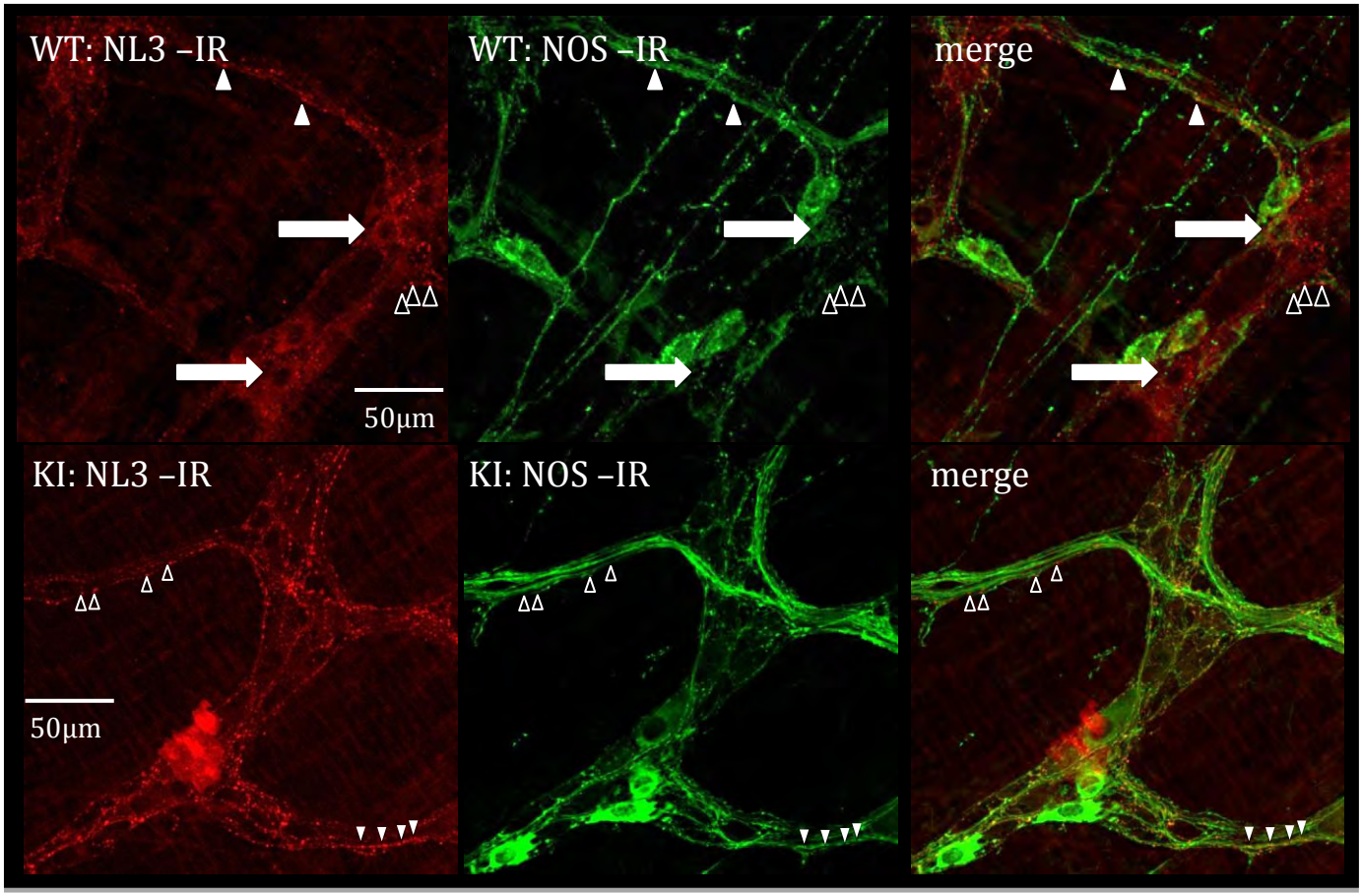

Figure 3: Localisation of NL3 and nNOS in the myenteric plexus of mouse jejunum. **Top:** Adult WT mouse jejunum myenteric plexus labelled for NL3 (red, 1:250) nNOS (green, 1:1000). **Horizontal arrows** show cell bodies labelled for NL3 but negative for nNOS. **Bottom:** Adult KI (NL3 Knock-in) mouse jejunum myenteric plexus labelled for NL3 (red, 1:500) and nNOS (green, 1:1000). **Open arrow heads** show presynaptic varicosities clearly labelled for NL3 but negative for nNOS. **Filled arrow heads** show presynaptic varicosities colocalizing NL3 and nNOS. Fewer NL3 immunoreactive cell bodies are visible in the KI.

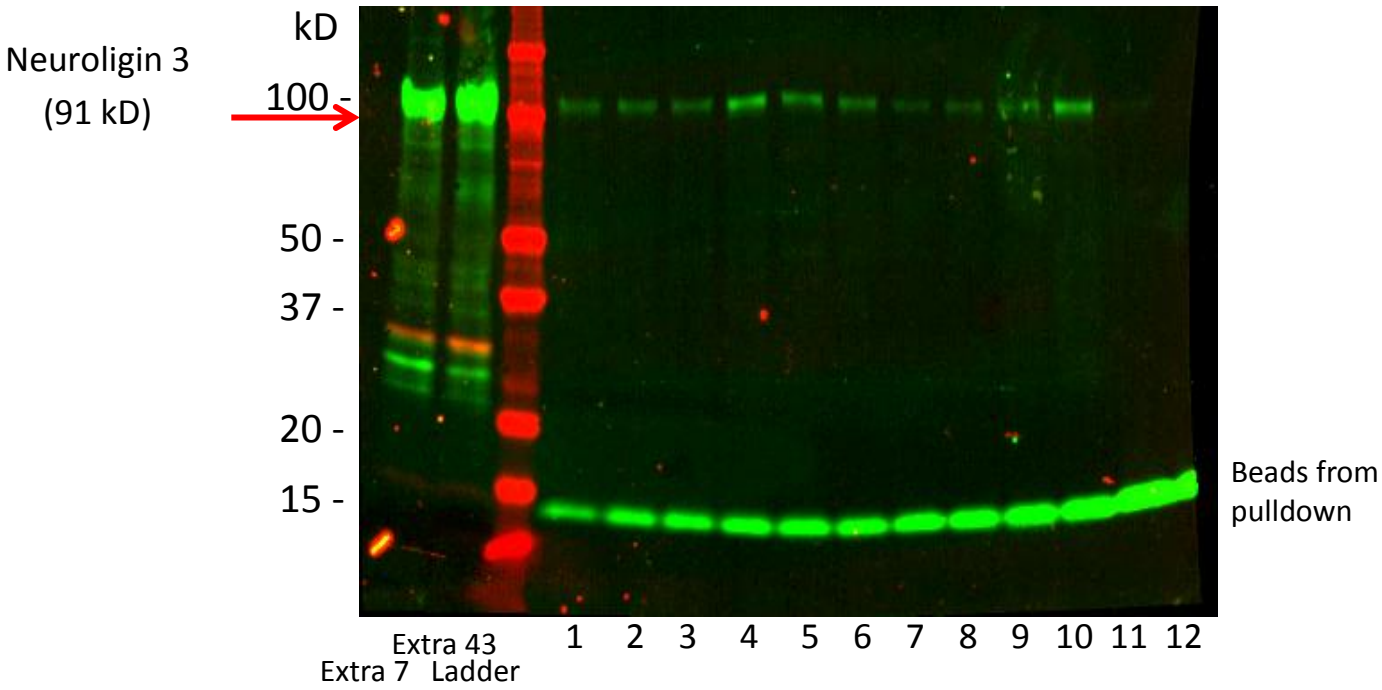

Figure 4: **Western blot showing specificity of the Neuroligin-3 antibody.** The Neuroligin-3 antibody (#129 113 rabbit polyclonal, Synaptic Systems) labels a 91kD protein (predicted weight of Neuroligin-3) in mouse hippocampal tissue (lanes 1-12).

Our findings that we cannot account for the altered efficacy of $GABA_A$ receptor blockade with an alteration in the number or proportion of GABA neurons (See report of PI Bornstein) indicate that there may be altered connectivity from or to these neurons. We have previously identified putative serotonergic synaptic contacts in the myenteric plexus using conventional confocal microscopy (eg using Z-stack images as illlustrated in **Figure 5)**; and shown that these differ between C57Bl6 and Balb/cJ mice with the latter being a strain with an autism behavioural phenotype (as reported in Neal et al., 2009). We will apply the same methods to analyse differences in enteric connectivity between $NL3^{R451C}$ and WT mice. Preliminary analyses of connections between GABA neurons and neurons reactive for nNOS (**Figure 5**) indicated that there were no such connections in either $NL3^{R451C}$ and WT colonic myenteric plexus, however the sample sizes thus far have been small (n = 10 in each case) and this conclusion requires confirmation. Furthermore, as some GABA and some nNOS neurons are interneurons in the colonic myenteric plexus, it is essential to examine other potential targets for each.

We are currently optimising immunohistochemical methods for use of the super-resolution microscope (OMX Blaze 3D-SIM microscope) which is a shared platform facility now available to our laboratory. This technique will enable image acquisition at an 8-fold increase in volume resolution compared to conventional confocal microscopy. Specifically we are resolving technical issues regarding appropriate secondary antibodies to ensure correct wavelength for avoiding bleaching during signal detection and envisage image acquisition to investigate the expression of the neuroligin 3 protein at the resolution of single synapses within the next month.

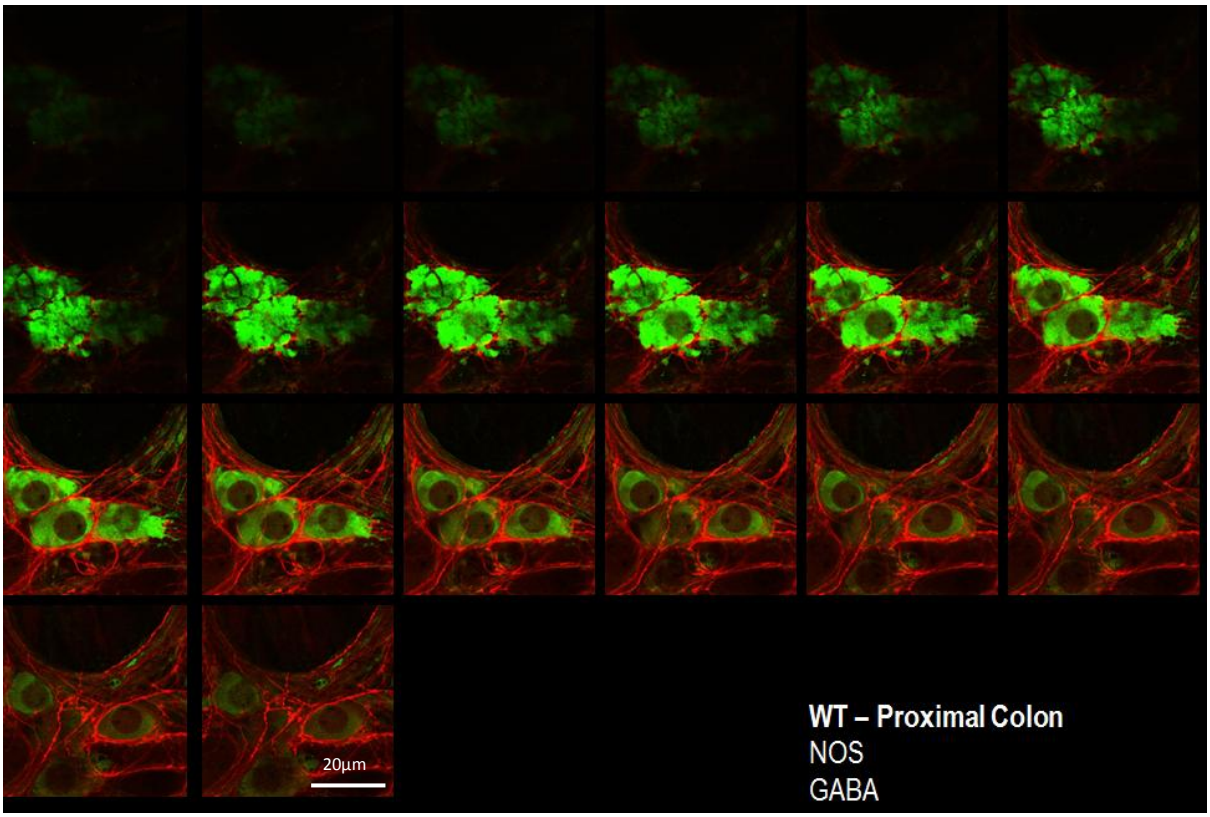

Figure 5: Adjacent Z-stack images for identification of close appositions in myenteric plexus using conventional confocal microscopy. Representative example of WT mouse proximal colon myenteric plexus labelled for nNOS (green) and GABA (red). Individual images of the confocal Z stack acquired by confocal microscopy will be analysed for close appositions in WT mouse myenteric plexus.

Our functional data included in the original application showed differential colonic motility in NL3 mutant vs WT in response to the 5HT3/4 receptor antagonist, Tropisetron (see PI Bornstein's report for extensions on this functional work). Because we observed altered responses to an antagonist at 5HT receptors, it is necessary to examine whether or not serotonergic connectivity is altered in NL3 mutant mice. This investigation forms part of Task 2c (NL3-expressing synapses). To examine for changes in serotonergic synapses we have undertaken triple labelling immunohistochemistry for the pan neuronal marker Hu which labels all neuronal cell bodies, neuronal NOS; a marker of cell soma and processes and serotonin (5HT) in the mouse colonic myenteric plexus (**Figure 6**). We show that serotonergic processes are present in the distal colon of both WT and NL3 KI mice (Ttask 2c) and we are currently quantifying these data. The exciting finding that NL3 appears to be expressed in presynaptic specializations in mouse myenteric plexus (i.e. see Figures 1 and 3) have required that we focus on characterizing the NL3 antiserum before proceeding further. However, the quantification of the 5HT synaptic immunohistochemistry will be completed in part 2 of the project and the optimisation of the triple labelling procedure that has already been achieved ensures that this work is well underway.

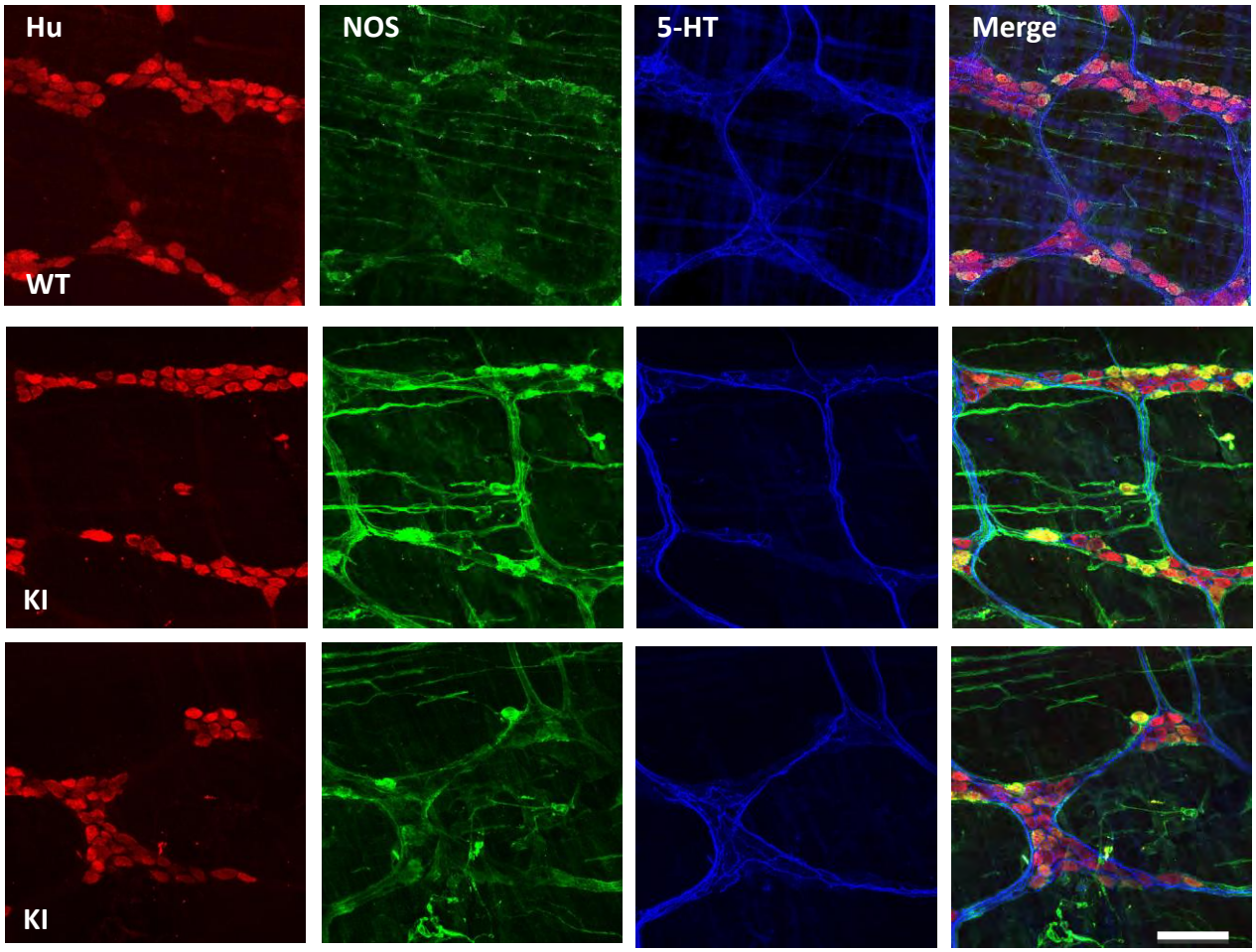

Figure 6: **Characterizing synaptic labelling for nNOS and 5-HT in the myenteric plexus of WT and KI mouse distal colon.** Representative images showing labelling for the pan neuronal marker Hu (1:5000; red), nNOS (1:1000; green), serotonin (5-HT; Immunostar, Hudson, USA; 1:2000; blue) in adult WT and NL3 mutant distal colon. Few serotonin immunoreactive cell bodies were observed however multiple serotonergic processes were visible, some colocalizing with nNOS. Immunocytochemistry for NOS labels a subset of neuronal cell bodies and processes. Labeling for 5HT shows neuronal processes and putative synaptic specializations (Scale bar: 100 µm).

Neuronal responses to electrical stimulation and GABA application

This work relates to **SOW Tasks 3a and 3b:** identifying functional classes of myenteric neurons; electrical stimulation and pharmacology and neurons responding to GABA.

Task 3. Identification of functional neuronal classes: PI Hill (22 months: months 3-24), 2-3 animals per week, total of 198 mice (66 Balbc/J, 66 WT, 66 NL3). (intracellular recording and biocytin labelling).
3a. Functional classes responding to GABA: intracellular recording, biocytin labelling, pressure ejection. (22 months: months 3-24). 30 cells (15 AH and 15 S cells) each from Balbc/J, WT and NL3 mice
3b. Electrical stimulation and pharmacology (Drugs: hexamethonium, PPADS, gabazine and granisetron) (22 months: months 3-24). A total of 90 cells; 30 cells (15 AH and 15 S cells) each from Balbc/J, WT and NL3 mice

The analysis of functional and morphological parameters in the myenteric plexus of the Balbc mouse colon has identified two main types of myenteric neuron (Nurgali et al., 2004). In addition electrophysiological and morphological findings from adult C57Bl6 mouse small intestine from the current laboratory (Foong et al., 2012) suggest that similar characteristics of myenteric neurons are present across mouse strains.

As reported by Nurgali and others, S neurons have a uniaxonal morphology typically display lamellar dendrites (**Figure 7**). S neurons show fast excitatory synaptic potentials (**Figure 8**) in response to fibre tract stimulation that are predominantly mediated via nicotinic receptor antagonists (Nurgali et al., 2004). In contrast, AH neurons have large smooth cell bodies and are predominantly multipolar (1-5 long smooth axon-like processes). AH neurons show an after hyperpolarization following firing of an action potential (**Figure 8;** Nurgali et al., 2004). Somata of AH neurons are predominantly large (> 30 uM in length) and show Dogiel type II morphology; i.e. smooth somata and processes.

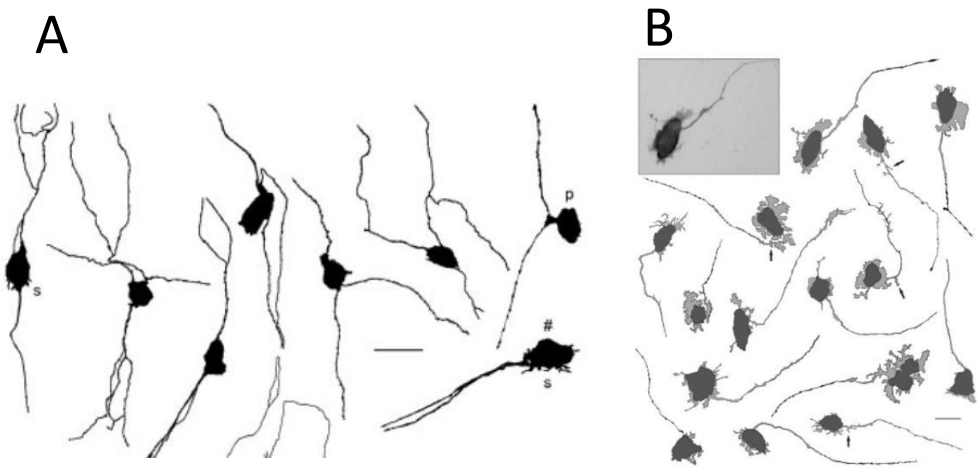

Figure 7: Morphology of and Dogiel type II (predominantly **AH neurons,** left panel) and Dogiel type I (predominantly **S neurons,** right panel) in adult mouse colon myenteric plexus (from Nurgali et al., 2004).

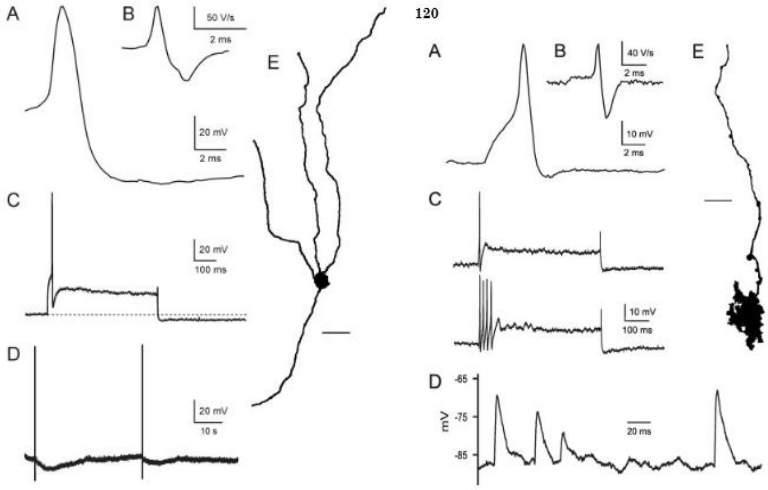

Figure 8: Electrophysiological responses of an AH neuron (left panel) and an S neuron (right panel) in adult mouse colon myenteric plexus (from Nurgali et al., 2004). Note after hyperpolarisation following the action potential in the AH neuron and spontaneous synaptic activity recorded from the S neuron.

Previous studies of the actions of GABA on myenteric neurons from guinea pig ileum concluded that it depolarized AH neurons, but had no effect on S neurons (Cherubini and North 1984a, Cherubini and North 1984b). However, there is no equivalent data from mouse enteric neurons, although $GABA_A$ mediated responses have been reported in a calcium-imaging study of cultured myenteric ganglia (Reis et al., 2006). Thus, our first goal was to determine if responses to GABA can be recorded from mouse myenteric neurons and if so whether responding neurons are AH or S neurons.

Thus far, a small sample of myenteric neurons from mouse colon has been studied and the data obtained confirm that GABA depolarizes a significant number of these neurons, as it does in the guinea pig myenteric plexus. However, unlike in the guinea pig small intestine, GABA depolarizes S neurons in the mouse colonic myenteric plexus, as identified by both electrophysiology (notably the presence of fast excitatory synaptic potentials; eg **Figures 9, 10 and 11**) and morphology (see **Figure 12**). This is an important finding, because in the guinea pig ileum GABA is confined to motor neurons and there is no reason to expect responses in other myenteric neurons, while in mouse colon GABA is also in interneurons. Identification of GABA responses in S neurons, which are interneurons and motor neurons, is thus an essential first step in identifying a function for GABA in the enteric circuitry and determining how this is altered in the $NL3^{R451C}$ mouse.

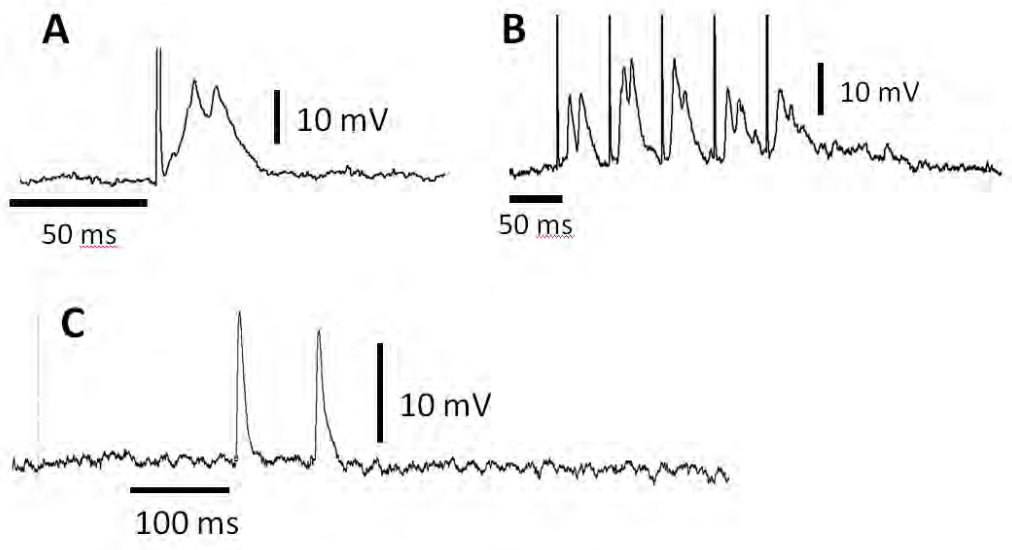

Figure 9: Fast excitatory postsynaptic potentials in a myenteric neuron in WT adult mouse colon. The initial resting membrane potential for this cell was approx. -30-35 mV which then increased to -85 mV. Traces show fEPSPs following single and multipulse stimulation. **A:** doublet response to single pulse stimulation (rmp -85mV). **B** same cell; multipeak response to multipulse stimulation. **C** This cell also consistently showed Proximal process potentials (PPPs) in response to local application of GABA (1mM; vertical line). Peak amplitudes of PPPs were 13.3mV and 13.1mV, Duration 0.0195 s and 0.0258 s, cell held at -50mV. GABA application was repeated at different holding potentials; each application resulted in PPP responses with increasing peak amplitude at more hyperpolarizing holding potentials. When the same cell was held at -70mV, local application of GABA resulted in two PPPs with peak amplitudes of 4.5 mV and 20.085 mV, duration 0.0195 s and 0.0207 s. From a holding potential of -80mV, GABA evoked 3 PPPs with peak amplitudes of 4.5 mV, 24.4 mV and 21.7 mV and durations of 0.0149 s and 0.0254s, 0.0254s. At -90mV, PPPs in response to GABA were recorded with higher mean peak amplitudes of 3.84 mV 28.6 mV and 26.5 mV and durations of 0.0143 s and 0.0326 s, 0.0322 s respectively. Mean duration of PPP responses for this cell was 23.03 ± 2 ms. This is likely an S neuron due to the presence of fEPSPs. Cell ID: 4_18-6-13.

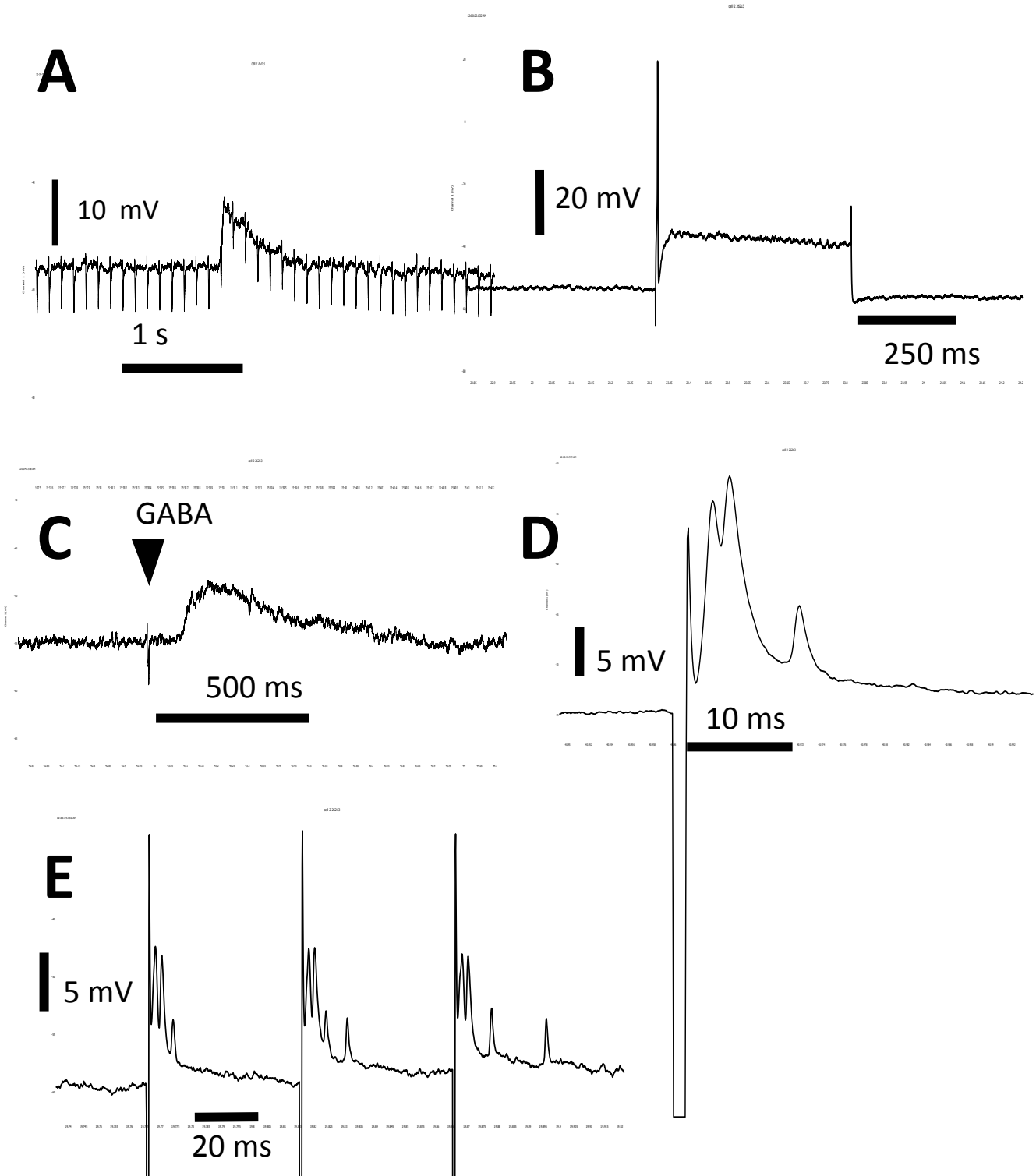

Figure 10: Response of a myenteric neuron to local application of 1mM GABA. A: GABA response -60 mV, peak amp 12.8mV **B**: Single action potential in response to 300pA step (500ms duration). **C**: Response to GABA 1mM, peak amplitude: 6.4 mV. This cell showed more than 10 responses to repeated GABA application (triangle indicates time of GABA application). This cell demonstrated fEPSPs a typical electrophysiological characteristic of S type neurons. Cell ID 2_26-02-13.

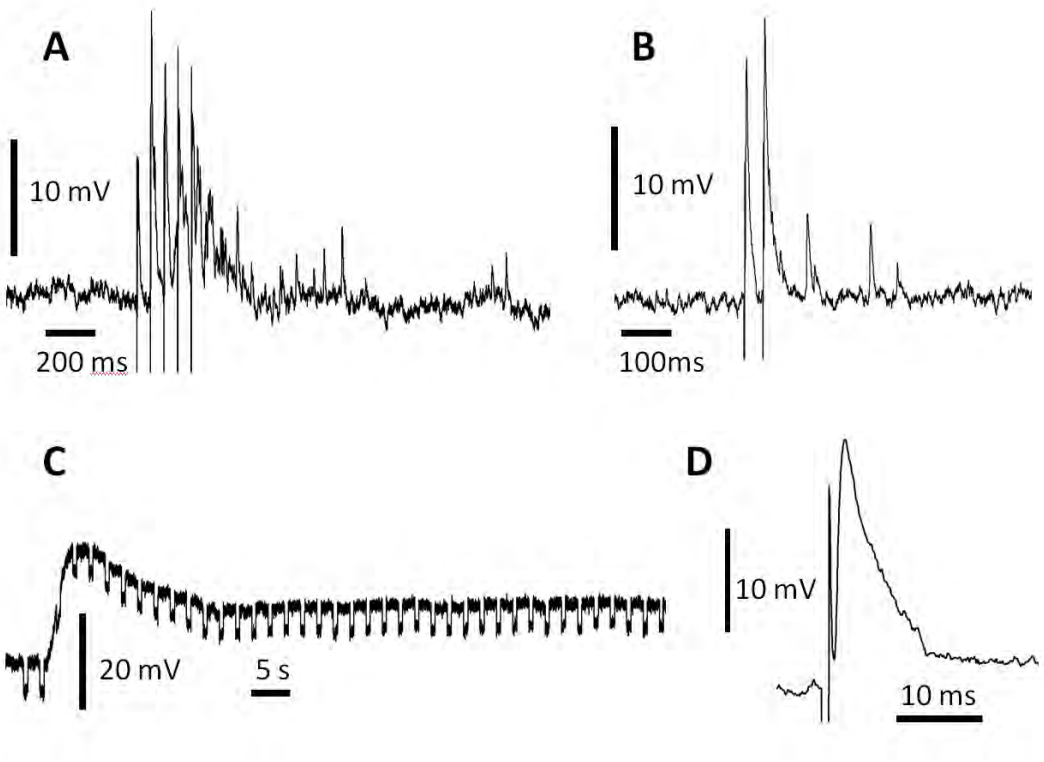

Figure 11 Spontaneous fast Excitatory Postsynaptic Potentials and GABA response in a myenteric neuron from a WT mouse A: response to multipulse stimulation demonstrating fEPSPs and spontaneous activity **B:** Multipulse (2 pulse) stimulation; amplitudes of first and second peaks respectively: 19.45 mV, 18.93 mV **C:** response to local GABA application **D:** fEPSP response to single pulse electrical stimulation (cell ID: 1_12-3-2013; Cell rmp = -47 mV). The fEPSPs seen both spontaneously and in response to electrical stimulation suggest that this cell is an S neuron.

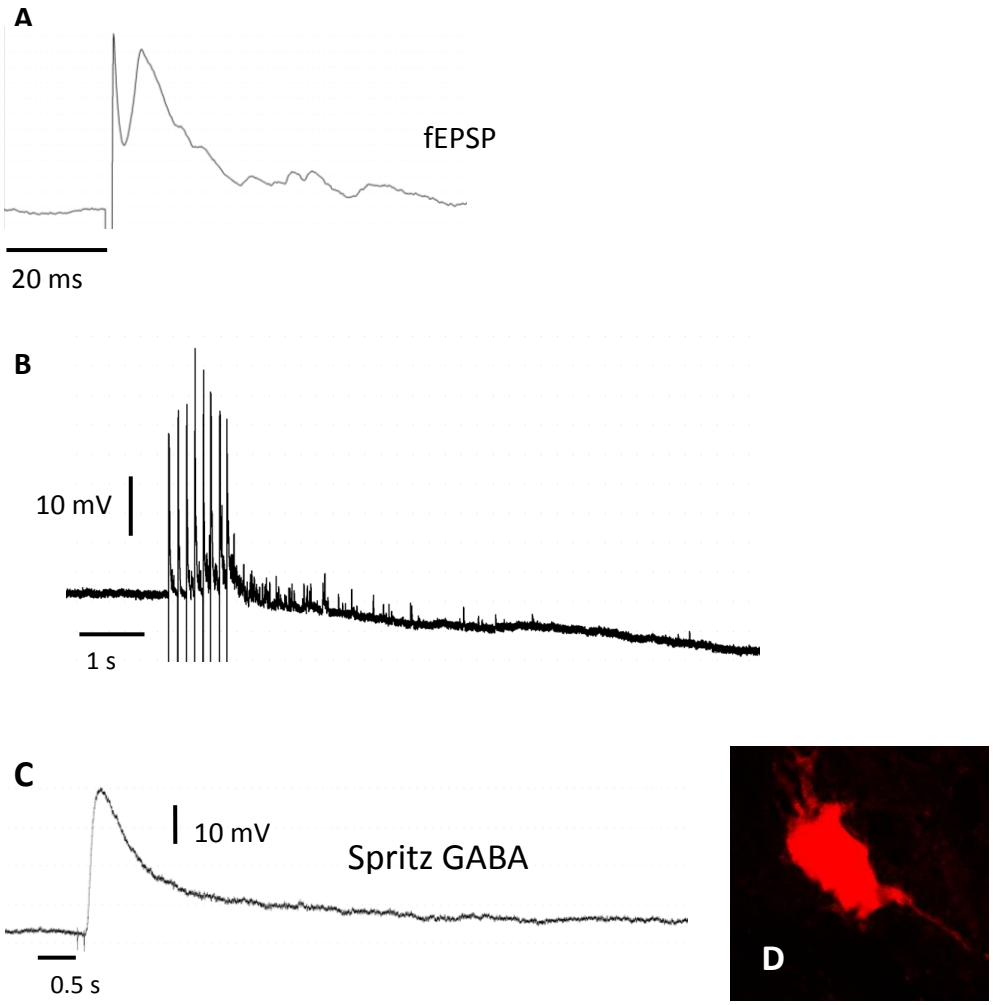

Figure 12: Intracellular recording and biocytin labelling of a myenteric neuron from a colonic preparation from an adult WT mouse. A: A fEPSP in response to a single pulse stimulation **B:** fEPSPs in response to a train of 10 pulses (20Hz). This cell also showed spontaneous excitatory activity following electrical stimulation. **C**: local application of 1mM GABA elicited a large depolarizing response (peak amplitude = 37.5 mV, rmp: - 67 mV). **D**: Cell labeled with Streptavidin Alexa Fluor 594 (1:200, Molecular Probes, Mulgrave, Vic Australia). The presence of spontaneous fEPSPs and lamellae dendrites adjacent to the cell body suggest that this is an S neuron. fEPSP: fast excitatory post synaptic potential. (cell ID: 9_26-08-2013)

Training related to this project

This task has involved training in microdissection, the intracellular recording technique and immunohistochemistry processing and image acquisition to determine functional neuronal classes in tissue preparations. As part of this project (Task 3), **PI Hill** has developed expertise in microdissection of fresh enteric tissue and intracellular recording from mouse myenteric plexus. As expected, initial experiments recording neuronal activity have been difficult but these are becoming easier and the rate of data acquisition is expected to increase over the remaining period of the project. **Ms Gracia Seger** has also developed expertise in tissue dissection, fixation and immunohistochemical techniques in order to examine NL3-expressing synapses (Task 2c) in the WT and Ki mice.

Key Research Accomplishments

- Localization of neuroligin 3 protein to a subset of neuronal cell bodies in the mouse colon and jejunal myenteric plexus.
- Demonstrated a species difference in neuroligin 3 protein expression whereby neuroligin 3 appears to be ubiquitously expressed in the guinea pig colon but restricted to a subset of neurons in the mouse colon and jejenum.
- Demonstrated that neuroligin 3 colocalizes with nNOS immunopositive neurons as well as a neuronal subset that is immunonegative for nNOS in the mouse.
- Localization of neuroligin 3 protein to presynaptic specializations in the myenteric plexus in both the colon and the jejunum of the mouse.
- Verified the specificity of the NL3 antiserum using Western Blot
- Demonstrated that S neurons in the proximal myenteric plexus of the mouse colon are depolarized in response to local application of GABA

Reportable Outcomes

1. **Manuscripts:**

- Argyropoulos A, Gilby KL, **Hill-Yardin EL***. Studying autism in rodent models: reconciling endophenotypes with comorbidities. Front Hum Neurosci. 2013 7:417. doi: 10.3389/fnhum.2013.00417. eCollection 2013. **corresponding author*(PDF attached – see appendices)
- **Hill-Yardin EL**, Hannan AJ Behav Neurosci. 2013;127(4):606-9. doi: 10.1037/a0033319. Translating preclinical environmental enrichment studies for the treatment of autism and other brain disorders: Comment on Woo and Leon (2013). (PDF attached – see appendices)
- *In preparation:* Burrows EL, Ellis M, Swaminathan M, Koyama L, Taher M, McKeown SJ, Parry LJ, Churilov L, Oezguen N, Savidge T, T O'Brien TJ, **Hill-Yardin EL**, Hannan AJ, **Bornstein JC**. Gastrointestinal dysfunction and aggression in a mouse model of autism. (PDF attached – see appendices)
- *Invited manuscript:* Swaminathan, M, Balasuriya, G, **Hill-Yardin EL., Bornstein, JC.** Video imaging of isolated colon preparations to study enteric nervous system function in mice *Journal of Visualized Experiments*

2. **Abstracts:**

- Gastrointestinal dysfunction mediated by $GABA_A$ receptors in the Neuroligin-3^{R451C} mouse model of autism. **Elisa L Hill-Yardin**, Melina Ellis, Numan Oezguen, Tor Savidge, **Bornstein JC**.Accepted for poster presentation at the Society for Neuroscience meeting San Diego, Nov **2013**.
- Altered inhibitory neurotransmission in the enteric nervous system of Neuroligin-3 R451C mice Bioautism 2013, Melbourne. Swaminathan M, Foong JPP, Ellis M, **Hill-Yardin EL**, **Bornstein JC.**
- Investigating neuronal subtypes and colonic motility in the neuroligin-3 R451C mouse model of autism. Swaminathan M, Foong JPP, Ellis M, **Hill-Yardin EL** and **Bornstein JC**. Australian Neuroscience Society (ANS), Melbourne, **2013.**
- The neuroligin 3 Arg451cys (NL-3) mouse model of Autism shows altered colonic function *in vitro*. Melina Ellis, Ali M. Taher, Sonja McKeown, **Elisa L. Hill, Joel C. Bornstein** Digestive Diseases Week, **2012,** San Diego**.**

3. Presentations/Seminars:

- 5th National Symposium on Advances in Urogenital and Gut Research Symposium, Monash Institute of Pharmacological Sciences, Melbourne, Friday, 29th November, 2013*, invited speaker.*
- 'Autism: a disorder of synapses' Frontiers in Physiology 3rd year undergraduate lecture series, The University of Melbourne 18th September 2013.
- "Autism in the gut" Olga Tennison Autism Research Centre (OTARC), LaTrobe University, Melbourne, 23 Aug 2013
- "Altered synaptic function in the neuroligin-3 model of ASD" Department of Physiology, Melbourne University, 7 May 2013

4. Media communications:

- Guest blog: "Robust models" Simons Foundation for Autism Research Initiative (SFARI; 11th Oct 2013): http://sfari.org/news-and-opinion/blog/2013/guest-blog-robust-models
- Article for The Conversation in response to a televised documentary regarding the role of gut bacteria in ASD: http://theconversation.edu.au/can-a-gut-bacteria-imbalance-really-cause-autism-9128
- Podcast interview with **Dr Kent Williams**, pediatric gastroenterologist for "Up Close, The research talk show" at Melbourne University http://upclose.edu.au 31 Jan 2013.
- Interviewed by Dr Shane Huntington 3RRR radio, Einstein-a-go-go Program, Highlights in autism research from the 2013 BioAutism meeting, 3 March 2013. http://www.rrr.org.au/program/einstein-a-go-go/

5. Funding applied for based on work supported by this award:

- Wenkart foundation PhD scholarship awarded to Niketa Archer from 2013 for investigating behavioural aspects of the NL3R451C mouse model. This work is not included in the current project but these experiments extend the *in vitro* findings in the NL3 R451C mouse model.

Conclusions

We present novel findings that the Neuroligin 3 protein is localized to a subset of myenteric neurons in the colon and jejunum of WT and NL3 mice. Expression patterns of NL3 in WT mice appear to differ between the small intestine and the colon with fewer somata labelling positively for NL3 in jejunal samples compared to the colon; but this finding requires quantification which we will complete in the second year of the project. Furthermore, the number and intensity of cell bodies labelling for NL3 in jejunum appears to be reduced in the mutant. We suggest that such a reduction in NL3 expression in the jejunum of mutant mice could be an indication of altered protein handling in the mutant (Comoletti et al., 2004). This observation is directly relevant to aim 2c and will be followed up using qPCR in the second part of the project and through quantitative Western blotting which is currently underway.

A major finding of this work is that NL3 is present in presynaptic specializations in mouse myenteric neurons both in the small intestine and in the colon. Based on the histological literature from CNS studies this was unexpected. However an increase in the frequency of inhibitory postsynaptic potentials in brain slices from $NL3^{R451C}$ mice was reported suggesting involvement of a presynaptic mechanism (Tabuchi et al., 2007) which would fit with our findings.

As described, we also labelled guinea pig myenteric plexus in order to confirm the specificity of the NL3 antiserum using funds from the NHMRC allocated to different projects. In the guinea pig we observed a different pattern of NL3 expression where all neurons appeared to express NL3 in addition to synaptic varicosities. Because this finding was unexpected we confirmed the specificity of the NL3 antibody by Western blot.

These unexpected findings requiring us to validate the specificity of the commercial antiserum, both through use in another species and via Western Blot, have slowed progress. In addition, finding NL3 expression in presynaptic specializations requires that the experimental plan be reassessed (ie presynaptic markers need to be used to enable further characterization) and as a result we are in the process of submitting an request to alter the experiments to be conducted under the SOW. Both the need to validate the antiserum and the unexpected findings of expression at presynapses have slowed progress such that part 2c of the project involving investigation of synapses labelled for NL3 has been delayed.

The important finding that NL3 labels a subset of myenteric neurons in the mouse needs to be quantified, as determining the neuronal subtypes expressing NL3 will enhance our understanding of how the R451C mutation influences gastrointestinal motility in these mice. Furthermore such studies will result in a greater understanding of the physiological role of NL3 in the ENS of wild type mice and this finding is already generating data that will be used in a future application to our basic research funding body the ARC (Australian Research Council) to study this. Given that these data contrast with earlier findings in dissociated hippocampal cells (Budreck and Scheiffele, 2007) these observations are important for the field in that clarification of NL3 expression in the CNS may also be warranted.

We further show that S Neurons in the myenteric plexus are responsive to local application of GABA. Although these results need to be explored further these findings will assist in identifying which neurons are involved in the GABAergic circuitry controlling the motility of the colon. This information is critical to understanding the underlying basis for our earlier finding of altered colonic motility in NL3 mice compared to WT littermates *in vitro* and in the second part of the project these findings will be expanded to further characterise these neurons.

The finding that NL3 is expressed at presynaptic specializations in the myenteric plexus contrasts with current literature which suggests that NL3 is exclusively found at the postsynaptic membrane. As a result, our findings have strong implications for the field and may assist in novel approaches to designing future experiments aimed at understanding the functional implications of this mutation and the design of potential therapeutic targets relevant to clinical applications.

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Q. Zhang *et al.*, Expression of neurexin and neuroligin in the enteric nervous system and their down-regulated expression levels in Hirschsprung disease. *Molecular biology reports* **40**, 2969 (2013).

Appendices

Task 2c: Localizing the neuroligin 3 protein in specific classes of enteric neurons

Summary of animals used for immunohistochemistry experiments:

Date	Mouse ID	Weight (g)	Sex	Genotype
13.08.13	210	26.72	Male	WT/O
14.08.13	170	24.16	Male	KI/O
27.08.13	245	31.53	Male	WT/O
28.08.13	223	29.17	Male	WT/O

Summary of antisera used:

Antiserum	Supplier	Cat. No.	Dilution
Rabbit Anti-Neuroligin 1	Santa Cruz	sc-50393	
Goat Anti-Neuroligin 2	Santa Cruz	sc-14089	
Rabbit Anti-Neuroligin 2	Synaptic Systems	129 203	
Rabbit Anti-Neuroligin 3	Synaptic Systems	129 113	1:1000 (Mouse) 1:500 (G.Pig)
Goat Anti-Neurexin 1	Santa Cruz	sc-14334	
Goat PSD-95	Santa Cruz	sc-6926	
Neuroligin 2 Control Peptide	Synaptic Systems	129 2P	
Neuroligin 3 Control Peptide	Synaptic Systems	129 3P	1:500 (G.Pig)
Sheep Anti NOS	Jackson West Grove USA		1:1000
Donkey anti Sheep Alexa 488 (green)	Life Technologies AustraliaInvitrogen		1:400
Donkey anti Rabbit 594 (green)	Life Technologies AustraliaInvitrogen		1:400

Task 3: Identifying functional classes of myenteric neurons

# mice used	Expt Date	Animal ID	genotype	sex	comments
1	14/03/2012	#156	Ki/o	♂	training : dissection and tissue preparation
2	19/03/2012	#36	Ki/o	♂	training : dissection and tissue preparation
3	20/03/2012	#37	Ki/o	♂	training : dissection and tissue preparation
4	27/03/2012	#30	Ki/o	♂	training : dissection and tissue preparation
5	27/03/2012	#28	wt/o	♂	training : dissection and tissue preparation
6	28/03/2012	#258	wt/o	♂	training : dissection and tissue preparation
7	29/05/2012	#262	Ki/o	♂	
8	25/02/2013	#45	Ki/o	♂	
9	26/02/2013	#46	Ki/o	♂	cell 1 and 2
10	4/03/2013	#44	Ki/o	♂	
11	12/03/2013	#52	wt/o	♂	cell 1 and 2
12	18/03/2013	#53	wt/o	♂	
13	4/06/2013	#158	Ki/o	♂	
14	12/06/2013	#102	wt/o	♂	
15	17/06/2013	#154	Ki/o	♂	2 cells
16	18/06/2013	#155	wt/o	♂	cell 4 S neuron "cell4 18613.adicht"
17	19/06/2013	#156	Ki/o	♂	cell 5, 6 and 7
18	20/06/2013	#157	Ki/o	♂	
19	21/06/2013	#163	wt/o	♂	
20	31/07/2013	#215	wt/o	♂	
21	13/08/2013	#230	wt/o	♂	
22	14/08/2013	#241	Ki/o	♂	
23	26/08/2013	#244	wt/o	♂	cell 9 probably S neuron "Elisa cell 9 260813.adicht" immunohistochemistry for biocytin
24	27/08/2013	#245	Ki/o	♂	cell 10 "cell10_270813.adicht', 'cell 11 270813.adicht' cell 12 270813.ad

Conference Abstracts

Accepted as a poster presentation at the **Society for Neuroscience meeting San Diego 2013**, meeting date November 9th-13th 2013. Poster session 531, Neural mechanisms associated with Autistic behaviors in Animals. Tuesday November 12th in Halls B-H.

Gastrointestinal dysfunction mediated by $GABA_A$ receptors in the Neuroligin-3^{R451C} mouse model of autism

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Gastrointestinal (GI) disorders are common in patients with autism spectrum disorder (ASD) and reduce quality of life. However, despite association of many genes influencing CNS synaptic function, the etiologies of these GI symptoms are unknown. Mutations in the neuroligin family of synaptic adhesion molecules are implicated in ASD disease progression. In order to identify underlying biological mechanisms, information on the expression of ASD candidate genes and the functional impact of the $NL3^{R451C}$ mutation in the enteric nervous system (ENS) is required. **Methods:** Human ASD-associated neuroligin mutations were structurally mapped using homology models generated for human NLG1, 2, 3 and 4Y and aligned to core structured regions of the NLGN4-X crystal structure. Protein-protein interfaces were predicted using the InterProSurf web server. Expression of neuroligins and neurexins was assessed in gut and brain cDNA using RT-PCR. Colonic migrating motor complexes (CMMCs) were analysed in isolated colon segments using video imaging techniques and spatiotemporal maps. CMMCs were assessed at baseline and in the presence of $GABA_A$ (bicuculline 10 µM and gabazine 10 µM) and $GABA_B$ (CGP 54626; 100 nM) receptor antagonists. **Results:** The structural domain harbouring R451C contained 5 of 7 identified mutation sites (Arg451 on NLGN3 and Asp429, Asp396, Val403 and Lys378 on Neuroligin 4X), closely juxtaposed to two protein-protein interface patches. We show expression of *Nlgn3*, related genes (*Nlgn1* and *Nlgn2*) and neurexin binding partners (*Nrxn 1* and *Nrxn 2*) in the mouse ENS. Further, both bicuculline (n = 16 WT, n = 16 $NL3^{R451C}$) and gabazine (n = 11 WT, n = 11 $NL3^{R451C}$) reversibly depressed CMMCs in NL3 mice compared to WT while CGP 54626 (n = 8 WT, n = 9 NL3) had no effect. Bicuculline reduced CMMCs in $NL3^{R451C}$ compared to WT colon (median difference: 6 contractions,

95% CI: 1, 12; p = 0.03). Gabazine also reduced CMMC frequency in $NL3^{R451C}$ mice compared to WT (median difference: 5 CMMCs, 95% CI: 2, 9; p = 0.009). In control conditions, CMMC frequency was identical in WT (n = 70) and $NL3^{R451C}$ (n = 69) tissues; in both WT and $NL3^{R451C}$ the median number of CMMCs was 4 (WT: 95% CI: 1, 11; $NL3^{R451C}$: 95% CI: 1, 15; median difference 1, 95% CI -2, 4; p = 0.509). **Conclusion:** Positional association of R451C with other clinically identified mutations suggest that a functionally conserved domain in the neuroligin family may be targeted in ASD. We show that the R451C knock-in mutation in the $NL3^{R451C}$ mouse model of ASD causes colonic motility dysfunction via a $GABA_A$ receptor mediated mechanism. These data implicate altered enteric synaptic function as a primary underlying cause of GI disorders in ASD.

Keywords: autism, gastrointestinal motility, mouse

Support:

This research was supported by a US Department of Defense Autism Research Program Idea Development Award (AR11034) to ELH-Y and JCB.

Abstract presented as poster presentation Australian Neuroscience Society,Gold Coast, QLD, Australia 2013:

INVESTIGATING NEURONAL SUBTYPES AND COLONIC MOTILITY IN THE NEUROLIGIN-3 R451C MOUSE MODEL OF AUTISM

Swaminathan M., Foong J.P.P., Ellis M., **Hill-Yardin E.L**. and Bornstein J.C.
Department of Physiology, University of Melbourne, Parkville Vic 3010.

Gastrointestinal problems are reported in up to 90% of Autism spectrum disorder (ASD) patients. Multiple gene mutations affecting synaptic function are associated with ASD. Neuroligin-3R451C mice express a missense mutation in the nlgn3 gene coding for the neuroligin-3 postsynaptic adhesion protein and show altered GABA-mediated colonic motility. Nitric oxide released from a subset of enteric neurons (immunoreactive for nitric oxide synthase; NOS) mediates tonic inhibition between colonic migration motor complexes (CMMCs). Some NOS neurons also express GABA. **Purpose:** To determine whether NOS-mediated colonic motility is altered in NL3R451C mice and if changes in motility correspond to altered proportions of GABA and/or NOS neurons in NL3R451C mice colon. **Methods:** Colons were isolated from C57/Bl6, NL3R451C and WT (C57/Bl6-sv129-J) mice. Effects of the NOS inhibitor L-Nitro-arginine (NOLA, 100μm) on colonic motility were examined using video imaging techniques. Immunohistochemistry for Hu (a pan-neuronal marker), GABA and NOS was conducted on whole-mount myenteric plexus preparations from NL3R451C and WT colon. **Results:** Application of NOLA increased CMMC frequency in C57/Bl6 colons (n=8; $p < 0.05$). Similarly, NOLA increased CMMC frequency in NL3R451C colons (n=9 in each group; $p < 0.05$). In contrast, CMMC frequency was unaffected by NOLA in WT littermates (n=9). Furthermore, when compared to WTs, C57/Bl6 colons showed increased CMMC frequency in response to NOLA ($p<0.05$).The proportion of GABA or NOS immunoreactive neurons in WT (n=3) and NL3 R451C (n=3) mice was unchanged. **Conclusion:** These results suggest that the NL3R451C synaptic mutation alters nitric oxide-mediated colonic motility and that sensitivity to NOLA is strain-specific. Altered colonic motility in NL3R451C is not due to altered GABA/NOS neuronal numbers.

Abstract presented as poster presentation at Digestive Diseases Week San Diego 2013

Nitric oxide mediated colonic motility is altered in the Neuroligin-3 R451C mouse model of Autism

Mathusi Swaminathan, Jaime Pei Pei Foong, Melina Ellis, Joel C Bornstein, Elisa L Hill-Yardin

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Purpose: Gastrointestinal function is compromised in up to 90% of patients with Autism spectrum disorder (ASD) and the underlying causes are unknown. $NL3^{R451C}$ mice express a missense mutation in the Nlgn3 gene coding for the postsynaptic adhesion protein, neuroligin-3 (NL3) found in ASD patients. We have previously shown that the excitatory component of organized colonic motility ($GABA_A$- and serotonergic- mediated) is perturbed in $NL3^{R451C}$ mice compared to wild type (WT) controls. Neuronal release of nitric oxide (NO) is responsible for tonic inhibition between colonic migrating motor complexes (CMMCs). Here, we examined whether the NO-mediated inhibitory component of spontaneous CMMCs is altered in $NL3^{R451C}$mice.

Methods: Full length colons were removed from $NL3^{R451C}$, WT littermates (control) and C57Bl/6 mice (comparison strain). Colonic motility was examined using video imaging techniques. After a 1 h control recording, the NO synthase (NOS) inhibitor L-Nitro-arginine (NOLA, 100µm) was applied into the superfusing solution and activity was recorded for 1 h. The NOLA was then washed out for 1 h while a further recording was made. The proportion of all myenteric neurons (marked by the pan neuronal marker, Hu) that contained GABA and/or NOS was investigated using immunohistochemistry. Myenteric neuronal density and connectivity between GABA+ and NOS+ neurons were examined.

Results: NOLA significantly increased CMMC frequency in $NL3^{R451C}$ ($n = 9$; $p = 0.031$), but not in WT littermates ($n = 9$; $p = 0.831$). NOLA also increased CMMC frequency in C57/Bl6 colons ($n = 9$; $p = 0.008$). No differences in neuronal density were observed between proximal (adjacent to cecum) and mid colon regions of WT and $NL3^{R451C}$ mice ($n = 3$ each; $p > 0.05$). Furthermore, there was no difference in the mean number of neurons per ganglion in the distal colon of WT and $NL3^{R451C}$ mice ($n = 3$ each; $p = 0.216$). In each colonic region, the proportions of neurons that were NOS+ and/or GABA+ were similar in WT and $NL3^{R451C}$ mice (GABA+; $p > 0.05$; NOS+; $p > 0.05$; GABA+/NOS+; $p > 0.05$; n=3 each). No

GABA+ appositions onto NOS+ neurons were observed, and GABA+ neurons rarely receive NOS+ appositions in $NL3^{R451C}$ mice.

Conclusions: $NL3^{R451C}$ mice show altered NOS-mediated colonic motility compared with WT controls. However, our findings also suggest a strain specific effect of NOLA on CMMC frequency. The changes in motility in $NL3^{R451C}$ colon are unlikely to be due to altered neuronal density or proportions of GABA or NOS neurons in $NL3^{R451C}$ mice suggesting that altered transmission at the level of the synapse is a likely explanation. These results provide the first evidence that a synaptic mutation associated with ASD can affect inhibitory elements of gastrointestinal motility.

Abstract accepted for oral presentation, BioAutism 2013 meeting, Melbourne, Australia.

Social communication in the Neuroligin 3$^{(R451C)}$ mouse model of autism.

Authors: Burrows EL[1], Howard ML[1], Kolbe S[2], **Hill EL**[3*], Hannan AJ[1*]

University/Institution: [1]Neural Plasticity Laboratory, Florey Institute of Neuroscience and Mental Health, University of Melbourne. [2]Department of Anatomy and Neuroscience, University of Melbourne. [3]Department of Physiology, University of Melbourne.

*both authors contributed equally to the study design/supervision of this work

Autism spectrum disorder (ASD) is a behaviourally defined condition in which patients show communication and social interaction deficits alongside the presence of repetitive behaviours. The cause of ASD is unknown. Mutations in synaptic function genes are present in a significant subset of ASD patients. A missense mutation (R451C) in the postsynaptic adhesion molecule neuroligin-3, has been identified in ASD patients. Mice containing the Neuroligin-3 ^{R451C} (NL3) mutation have been previously characterized as a mouse model of ASD and we report extensive phenotyping with behavioural assays established to evaluate core and associated autistic-like traits, including tests for social abnormalities, ultrasonic vocalizations, perseverative and stereotypic behaviours and anxiety. We have identified a novel aggression phenotype in NL3 mice, a comorbidity present in 70% of ASD patients. This trait was reversed by treatment with the clinically effective antipsychotic, risperidone, a criterion for validating animal models of autism. In addition to displaying heightened aggression towards male intruder mice, NL3 mice also display aggression towards females during mating, a behaviour not seen in WT mice. We have further explored the social cognition behavioural phenotype of NL3 mice by investigating social communication by ultrasonic vocalizations. Ultra-sonic vocalizations were examined in adult mice in a number of different social contexts, including ano-genital sniffing in direct interactions with female mice and during presentation of olfactory cues from conspecifics. These studies will assist understanding of biological mechanisms contributing to ASD and contribute to the design of novel therapeutic treatments for ASD patients.

Biographical Sketch

Provide the following information for each individual included in the Research & Related Senior/Key Person Profile (Expanded) Form.	
NAME: HILL, Elisa Llewellyn	POSITION TITLE: Research Fellow, University of Melbourne

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
University of Melbourne, Australia	BSc (Hons)	1994	Cell Biology
University of Melbourne, Australia	PhD	2001	Neuroscience
École Supérieure de Physique et de Chimie Industrielles, Paris		2001-2004	Postdoctoral research
Howard Florey Institute, Melbourne		2006-2010	Postdoctoral research
Department of Medicine, University of Melbourne		2010-2011	Postdoctoral research
Department of Physiology, University of Melbourne		2012-	Postdoctoral research

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

Positions and Employment

1995: Technical Officer, School of Dental Science, Melbourne University, Australia

1995-1996: Research Assistant, Dept of Anatomy & Cell Biology, Melbourne University, Australia

2001-2004: CNRS Postdoctoral Fellow (CNRS Chargé de Recherche Associé, CRA 4). Laboratoire Neurobiologie et Diversité Cellulaire, Ecole Supérieure de Physique et de Chimie Industrielles, Paris, France.

2006–2008: NHMRC Howard Florey Centenary Postdoctoral Fellow, Howard Florey Institute, Melbourne, Australia

2006-2010: Research Fellow: Department of Anatomy & Cell Biology, University of Melbourne, Australia

2008 -2010: NHMRC Senior Research Officer: Howard Florey Institute, Melbourne, Australia

2010-2011: NHMRC Research Fellow: Department of Medicine, University of Melbourne, Australia

2012-: US DoD CDMRP Autism Research Program Research Fellow, Department of Physiology, University of Melbourne

Honors

1996: Australian Postgraduate Award Scholarship to research "Neurochemical Signatures in Mouse Neocortex" during my PhD.

2000: Recipient of the Ayse Berke and Liana Colvill Award, Howard Florey Institute. The award is dedicated to the most outstanding PhD student (from a group of approximately 40 students in 2000)

2000: Recipient of an AMRAD Student Travel Award

2001: Fondation de Recherche Medicale Fellowship (FRM, France)

2002: Centre National de la Recherche Scientifique Poste Rouge Fellowship (CNRS, France)

2006: NHMRC, Howard Florey Centenary Fellowship, to study "Thalamocortical relay circuits in a novel epilepsy mouse model"

2008: Recipient of The Caitlin's Fund Travel Award for Epilepsy Research

Other experience and Professional Memberships:

1995-present:	Member, Australian Neuroscience Society
2000-2002/2013:	Member, Society for Neuroscience
2001 – present:	Member, Fondation Kastler
2006- present:	Member, Australian Society for Medical Research (ASMR)
2007- present:	Member, Autism Victoria, Australia
2004-2005:	Member, Federation of European Neurosciences Societies (FENS)
2006 – present:	Reviewer for Clinical and Experimental Pharmacology and Physiology
2009:	Organiser, Florey Neuroscience institutes Symposium, Melbourne
2011:	Lead Organiser, BioAutism 2011 and 2013 conferences, Melbourne. This meeting was the first in Australia to engage professionals and multidisciplinary researchers with a focus on basic research into autism.
2013:	Reviewer for Frontiers in Human Neuroscience

Publications in past 3 years (2010 onwards):

1. Studying Autism in rodent models: reconciling endophenotypes with comorbidities. Argyropoulos A, Gilby K, **Hill-Yardin EL** ******corresponding author***.** Front Hum Neurosci. 2013 Jul 25;7:417. doi: 10.3389/fnhum.2013.00417. IF 2.9.

2. Translating preclinical environmental enrichment studies for the treatment of autism and other brain disorders: Comment on Woo and Leon (2013). **Hill-Yardin** EL, Hannan AJ. Behav Neurosci. 2013 Aug;127(4):606-9. doi: 10.1037/a0033319. *Invited commentary*

3. **Hill EL,** Hosie S, Mulligan R, Richards K, Reid C, Baram TZ, Jones M, Steven Petrou. Transient temperature elevation causes a prolonged increase in GABA-mediated inhibition in cortical neurons in the Gabrg2R43/Q43 mouse model of familial epilepsy. **Epilepsia**. 2011 Jan;52(1):179-84.IF 4.052).

4. Wimmer V, Reid C, Mitchell S, Richards K, Scaf B, Leaw B, **Hill E**, Royeck M, Horstmann M-T, Cromer B, Davies P, Xu R, Lerche H, Berkovic S, Beck H, Petrou S. Axon initial segment dysfunction in human genetic epilepsy with febrile seizures plus. **J. Clin. Invest.** 2010 Aug 2;120(8):2661-71. IF 16.559

NB: Career break from end 2007 – mid 2008

Representative earlier publications relevant to this application (analysis of cellular diversity).

5. **Hill EL,** Schweitzer P, Gallopin T, Férézou I, Cauli B, Rossier J, Lambolez B. Localization of Functional CB1 Receptors in Neocortical Neurons **J Neurophysiol**. 2007 97(4):2580-9. IF=3.853

6. Ferezou I, **Hill EL**, Cauli B, Gibelin N, Kaneko T, Rossier J, Lambolez B. Extensive overlap of mu-opioid and nicotinic sensitivity in cortical interneurons. **Cerebral Cortex**. (2007). 17(8):1948-57 IF=6.187

NB: Career break from April 2004 – end 2005

7. Férézou I, Cauli B, **Hill EL**, Hamel E, Rossier J, Lambolez B. Serotonergic synaptic excitation of neocortical VIP/CCK interneurons mediated by 5-HT3 receptors. **J Neurosci**. 22(17):7389-97 (2002) IF=7.506
8. **Hill, E**, Kalloniatis M, and Tan S-S. Cellular diversity in mouse neocortex revealed by multispectral analysis of amino acid immunoreactivity. **Cerebral Cortex**, 11:679-690 (2001). IF=6.187 **This research was featured as the full page front cover illustration of the journal*
9. **Hill, E**, Kalloniatis M, and Tan S-S. Glutamate, GABA and precursor amino acids in adult mouse neocortex: cellular diversity revealed by quantitative immunocytochemistry. **Cerebral Cortex**, 10:1132-1142 (2000) IF=6.187

Leaves of Absence (total 2.5 years):

- Maternity Leave: April 2004 – January 2006 (21 months)
- Maternity Leave: October 2007 – April 2008 (7 months)
- Carer's Leave: April 2008 – June 2008 (2 months)
- Ongoing care for a child with special needs (autism)

Contribution to training:

Supervisor of twelve post-graduate students, one has completed their PhD, five completed honors (3 with high distinction, 2 awaiting results), 3 current PhD, 3 current Masters.

Research support

- NHMRC (Australia) Project Grant (Hannan, Hill, Malone) of AU$563,000 'Pathogenesis and therapeutic modulation of aggressive behaviour in a mouse model of autism spectrum disorder' (2013-2015)
  Role: Chief Investigator B

- NHMRC (Australia) Project Grant of AU$292,250, as CIA (sole investigator), entitled 'Mechanisms underlying generation of Febrile Seizures in mouse models of human familial epilepsy', (2008-2011)
  Role: Principal Investigator

- Monash Institute of Pharmacological Sciences: Faculty large grants scheme (Malone, Hill, Hannan). 'Sensorimotor gating, aggression and neuronal inhibition in a mouse model of autism'. AU$15,300 (2011)
  Role: Principal Investigator

- Brain Foundation Epilepsy Award (French, Hill and Zheng). 'How do anti-epileptic drugs work?' AU$40,000 (2011) Role: Co-Investigator

Gastrointestinal dysfunction and aggression in a mouse model of autism

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Abstract

Gastrointestinal (GI) motility dysfunction and aggressive behavior are common in patients with autism spectrum disorder (ASD) and reduce quality of life. However, despite association of many genes influencing CNS synaptic function, the etiologies of ASD symptoms are unknown. Mutations in the neuroligin family of synaptic adhesion molecules are implicated in ASD disease progression. Here we demonstrate that the ASD causing missense mutation (R451C) in neuroligin-3 (NL3) is located in a highly conserved structural domain preferentially targeted by clinically identified neuroligin mutations. Importantly, *Nlgn3*, related genes (*Nlgn1* and *Nlgn2*) and neurexin binding partners (*Nrxn 1* and *Nrxn 2*) were robustly expressed in the mouse enteric nervous system (ENS), and the R451C knock-in mutation in the $NL3^{R451C}$ mouse model of ASD caused colonic motility dysfunction via GABAergic ($GABA_A$) receptors. The proportion of neurons immunoreactive for GABA was unchanged in $NL3^{R451C}$ mice suggesting that altered motility is unlikely due to abnormal cellular proliferation during development. $NL3^{R451C}$ mice displayed an aggressive phenotype that was reversed by risperidone administration, verifying the utility of this model for ASD research. These data implicate altered enteric synaptic function as a primary underlying cause of GI disorders in ASD. Identification of specific ENS receptor subtypes mediating altered GI function may assist in the development of therapeutic targets for patient treatment.

Keywords: autism, gastrointestinal motility, mouse, neuroligin-3, aggression

Introduction

GI dysfunction and aggressive behavior are serious and commonly observed traits in patients with ASD. GI dysfunction is not currently included in the diagnostic criteria of ASD because definitive evidence for such involvement being intrinsic to ASD is lacking[1]. However, GI symptoms are reported in up to 90% of ASD patients, with chronic constipation being the most prominent clinical presentation[2, 3]. Aggression is observed in 70% of ASD patients[4] and has a major negative impact on patient quality of life. Understanding the mechanisms underlying GI dysfunction and aggression will accelerate the development of therapies for these co-morbid symptoms, for which there are currently limited treatments. In a bid to achieve this, we investigated gastrointestinal motility and aggression in the $NL3^{R451C}$ mouse model of ASD.

Many mutations in synaptic adhesion proteins including multiple members of the neuroligin family and their binding partners, the neurexins, are implicated in ASD[5-9]. NL3 is expressed at both excitatory and inhibitory synapses in the CNS[10]. Although the precise role of NL3 is unclear, neuroligins and neurexins function within a large network of proteins, including PSD95 and the SHANK family of cytoplasmic scaffolding proteins that influence a broad range of synaptic functions, such as vesicular and receptor recycling, neurotransmitter release and synaptic scaffolding[9, 11]. The NL3 R451C mutation was identified in two siblings with ASD[6] and results in a 90% reduction in the level of neuroligin-3 protein at the postsynaptic membrane in central neurons[12]. Two mouse lines expressing the R451C mutation show altered excitatory versus inhibitory neurotransmission signalling, whereby GABAergic inhibitory transmission is increased in somatosensory cortical slices[13], alongside increased glutamatergic transmission in the CA1 region of the hippocampus[14]. Furthermore, GABA signalling is enhanced during development in hippocampal CA3 neurons[15]. These data

confirm that synaptic function is altered in these mice and that the R451C mutation contributes to a 'gain of function' of CNS neurotransmission.

The enteric nervous system (ENS) is largely autonomous and many of its functions are seen in completely isolated tissues. The ENS contains intrinsic sensory neurons, interneurons, secretomotor, excitatory and inhibitory motor neurons, which generate complex motor activity[16]. Many pre and postsynaptic proteins required for typical CNS function are also present in the ENS[17-19], but ENS function has not been assessed in genetic mouse models of ASD[20]. The mouse colon exhibits highly stereotyped repetitive motor patterns (colonic migrating motor complexes; CMMCs) that depend on neural activity, when isolated from the CNS and all humoral influences *in vitro*[21], thus making this an ideal assay system for studying disturbances of ENS function resulting from synaptic dysfunction independently of CNS activity. GABA is found in a subset of interneurons and some motor neurons in the mouse colonic ENS[22] and has been implicated in synaptic transmission within the enteric neural circuitry[23]. In order to investigate GABAergic neurotransmission we measured CMMCs in the $NL3^{R451C}$ mice.

Impairment in social interaction, an endophenotype relevant to social impairments observed in ASD patients is a key criterion for animal models of autism. The precise nature of the changes in social behavior in $NL3^{R451C}$ mice are unclear as previous studies have yielded ambiguous results[13, 14, 24-26]. In order to further characterize the $NL3^{R451C}$ model of autism, we investigated aggressive behavior in these mice and the effects of the atypical antipsychotic risperidone, which is used to treat aggression in ASD patients. We show for the first time that a gene mutation associated with autism alters GI motility in mice and demonstrate that this NL3 mutation is associated with increased aggression that can be reversed by risperidone.

Materials and Methods

Animals. B6;129-Nlgn3tm1Sud/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine USA) and maintained to generation F9 on a hybrid Sv129/C57Bl6 background. $NL3^{R451C}$ and WT animals were derived by mating heterozygous females with $NL3^{R451C}$ males, which produced 50:50 WT and $NL3^{R451C}$ male offspring (Y/+ and Y/R451C) and genotyped as described[13]. Experimental animals were weaned at 4 weeks of age and housed in groups of four per cage with food and water available *ad libitum*. The holding room was maintained on a 12:12 hr light/dark cycle with lights on at 7 a.m. and at an ambient temperature of 20 ± 1°C. All procedures were approved by the Florey Neuroscience Institutes and The University of Melbourne Animal Ethics Committees.

Structural mapping of human ASD-associated neuroligin mutations. Homology models for human NLG1, 2, 3 and 4Y were retrieved from the Swiss-Model Repository as previously described[27-29]. These models were reliable as indicated by very high sequence to template identity (>81%). Core structured regions of the NLGN4-X crystal structure and models were aligned using Molmol[30]. Surface exposed protein-protein interface patches for NLGN3 were predicted using the InterProSurf web server[31].

RT-PCR. Total RNA was isolated from gut and brain tissues, and cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mix with random hexamers (Supplementary Methods). Expression of neuroligins and neurexins was assessed using RT-PCR with intron-spanning specific primer pairs designed from full-length sequences of mouse neuroligin and neurexin genes (Supplementary Table S1).

GI motility experiments. Adult male $NL3^{R451C}$ and WT mice (25-35g) were euthanized by cervical dislocation. The entire colon (~5 cm) was dissected, flushed and cannulated in an organ bath superfused with physiological saline (at 37^{o}C, 95%O_2/5% CO_2, flow: 5 ml min^{-1}).

After a 30 min equilibration, video recordings were acquired using a Logitech Quickcam pro 9000 camera (30 fps, 640 x 840 resolution) in order to measure contractile motor activity for a duration of 3 hrs. Motor activity was recorded at baseline (1 hr), with bath-applied antagonist (1 hr) and during washout (1 hr). Pseudocolored spatiotemporal maps of motor patterns were created and motor pattern frequencies were quantified using in-house software[32]. Bicuculline (Tocris), gabazine (Sigma) and CGP 54626 (Tocris) were diluted in saline.

Immunohistochemistry. Wholemount colonic myenteric plexus and longitudinal muscle (LMMP) preparations from WT and NL3 mice were processed for immunohistochemistry using antisera raised against Hu (a pan neuronal marker, a gift from Dr. V. Lennon, ,Mayo Clinic, USA) and GABA (Sigma-Aldrich, Castle Hill, Australia). See Supplementary Methods.

Resident-intruder test. Male resident mice were isolated for 1 week, during which their home cages were not changed. Aggressive behaviors in 3 month-old mice were monitored during four 5 min test exposures to 8 week old C57BL/6 male intruder mice conducted over 4 days. Latency to first attack, attack incidence and non-aggressive social interactions were recorded from videotapes of each test session. Non-aggressive social interactions were defined as sniffing, climbing on and grooming the intruder. Trials were aborted if the experimenter observed tufts of hair being removed from either animal. $NL3^{R451C}$ mice were injected intraperitoneally with a non-sedative dose of risperidone (0.05 mg/kg; Sigma) or saline 15 minutes prior to testing (Supplementary Figure S1). Testing was conducted in the light cycle between the hours of 9am-5pm, blind to genotype, and drug treatment and treatment group assignment was randomized.

Statistical analysis: See Supplementary Methods

Results

More than one hundred rare gene mutations are associated with ASD, and many of these affect synaptic function[5, 9]. Several clinically identified mutations target the neuroligin family, which is structurally conserved in 3D topology (Figure 1A, B and Supplementary Video S1). We structurally mapped known human neuroligin missense mutations to establish whether positional correlates exist with the R451C mutation in *Nlgn3*, because this is associated with ASD symptoms in humans and mice. The structural domain harbouring R451C also contained 5 of 7 other identified mutation sites (Arg451 on NLGN3 and Asp429, Asp396, Val403 and Lys378 on Neuroligin 4X), closely juxtaposed to two protein-protein interface patches (Figure 1C and Supplementary Video S1). All of these mutation sites were highly conserved in all neuroligin isoforms, and across species in human, mouse and rat structural variants. Positional association of R451C with other clinically identified mutations suggest that a functionally conserved domain in the neuroligin family may be targeted in ASD.

The idea that a synaptic mutation could impact on the periphery by directly affecting gut function in ASD is untested[33]. We first showed that relevant neuroligins and neurexins are expressed in the GI tract of mice (Figure 2). Transcripts for expression of *Nlgn1*, *Nlgn2*, *Nlgn3*, *Nrxn1* and *Nrxn2* were identified in adult mouse duodenum (Figure 2A, Supplementary Table S1) and predominantly expressed in the myenteric plexus/longitudinal muscle of both duodenum and colon (Figure 2B, Supplementary Table S1). Thus, mutations in these genes associated with ASD will be present in the ENS. Hence, we investigated if the R451C mutation in *Nlgn3* identified in ASD patients alters GI behavior in the $NL3^{R451C}$ mouse.

$NL3^{R451C}$ mice show altered $GABA_A$ receptor mediated colonic motility

As $NL3^{R451C}$ mice show enhanced GABAergic neurotransmission in the CNS[13, 15], we tested if enteric neural circuits in $NL3^{R451C}$ colon show altered involvement of GABA. In control conditions, CMMC frequency was identical in WT and $NL3^{R451C}$ tissues (Figure 3D); in WT mice the median number of CMMCs per 15 minute recording period was 4 (95% CI: 1, 11) and in $NL3^{R451C}$ 4 (95% CI: 1, 15) (median difference 1, 95% CI -2,4; p = 0.509). When the neural network regulating CMMCs was challenged by exposure to bicuculline ($GABA_A$ antagonist 10 µM, Figure 3B, C, E, H), $NL3^{R451C}$ mouse colon exhibited significantly fewer CMMCs than WT colon (median difference over a 1 hr duration: 6 contractions, 95% CI: 1, 12; p = 0.03; Supplementary Video S2). In an independent sample treated with another $GABA_A$ antagonist, gabazine (10 µM, Figure 3B, C, F, I), the number of CMMCs in $NL3^{R451C}$ mice was also reduced compared to WT (median difference: 5 CMMCs, 95% CI: 2, 9; p = 0.009). In contrast, CMMC numbers were unaffected by the $GABA_B$ antagonist CGP 54626 (100 nM, Figure 3B, C, G, J; median difference: -1 CMMC, 95% CI: -9, 6; p = 0.809), suggesting that a $GABA_A$ receptor specific mechanism is involved. Together, these data demonstrate altered $GABA_A$ receptor-mediated signalling in $NL3^{R451C}$ colon and provide the first evidence that a gene mutation associated with ASD produces GI dysfunction via an effect in the ENS.

The percentage of neurons immunoreactive for GABA is unchanged in $NL3^{R451C}$ colon

Perturbations in enteric neural serotonin during enteric development lead to changes in both number of neurons and proportion of GABA neurons, along with significant disturbances in colonic motility[34]. In order to examine neuronal density, neuronal cell bodies (immunoreactive for the pan neuronal marker Hu) were counted in proximal, mid and distal colon preparations (Supplementary Methods). We then assessed whether the $NL3^{R451C}$ mutation alters the percentage of neurons immunoreactive for GABA in the proximal, mid and distal colon. No difference in the mean density of neurons was detected in proximal and

mid colonic regions taken from WT and $NL3^{R415C}$ mice (neurons per mm^2 in proximal colon: 317 and 315; mid colon: 314 and 306; WT and NL3, respectively). In the proximal colon, the mean difference in neuronal numbers was -2 (95% CI: -128, 124) and in the mid colon; -8 (95% CI: -135, 118; p = 0.910). Since neuronal populations are comparatively sparsely distributed in the distal colon, a total of 20 ganglia were counted from each preparation to compare the mean number of neurons per ganglion between WT and $NL3^{R451C}$ mice. We found that the mean number of neurons per ganglion in the distal colon were similar between WT and $NL3^{R451C}$ mice (24.1 21.4 respectively; mean difference: -2.58; 95% CI: -7.5, 2.3; p = 0.216).

Although the total neuronal numbers were unchanged in WT compared to $NL3^{R451C}$ colon, the proportions of neuronal subsets could contribute to the functional changes observed in $NL3^{R451C}$ colon in response to $GABA_A$ receptor antagonists. Therefore we assessed for regional and genotype differences between the proportions of GABA immunoreactive neurons (GABA neurons) in WT and $NL3^{R451C}$ colon as a percentage of Hu immunoreactive neurons. There were no significant differences between genotypes in the percentage of GABA neurons in the proximal colon (WT: 12, $NL3^{R451C}$: 10; mean difference -1.8; 95% CI: -10, 6.5,) or in the mid colon (WT: 13, $NL3^{R451C}$: 17; 95% CI: -5, 12). Similarly, there were no genotype differences in the percentage of distal colonic GABA neurons (WT: 28, $NL3^{R451C}$: 27; 95% CI: -9, 7) in WT and $NL3^{R451C}$ mice. When data from all regions of the colon were compared, the percentage of GABA neurons was higher in the distal colon compared to the proximal and mid colonic regions ($p < 0.0001$) but no differences were observed between WT and $NL3^{R451C}$ mice (p = 0.953). Thus, the increased susceptibility of CMMCs to $GABA_A$ receptor blockade in $NL3^{R451C}$ mice is unlikely due to alterations in numbers of GABA neurons. In light of these data we suggest that the functional changes observed in $NL3^{R451C}$ mice may be occurring at the level of enteric GABA synapses.

NL3^{R451C} mice display heightened aggression

To clarify the nature of the social behavioral phenotype of mice expressing the R451C mutation, we tested for endophenotypes relevant to social impairments observed in ASD, particularly aggression. Using the resident-intruder assay we found heightened levels of aggression in NL3^{R451C} mice towards a novel intruder mouse compared to WT littermates. Two NL3^{R451C} animals were excluded from testing on the first day due to extreme aggression towards the intruder. NL3^{R451C} mice were 8 times more likely to initiate the first attack when confronted by an intruder mouse (95% CI: 1.52, 41.94; p = 0.014) at any point over the 300 s observation period (Figure 5A). Enhanced aggression in NL3^{R451C} mice was also reflected by an increase in the incidence of attacks (ratio of expected number of attacks = 5.63; p = 0.029; 95% CI: 1.19, 26.61; Figure 5B). To explore non-aggressive interactions, mice were scored for the duration of time that they engaged in anogenital sniffing, climbing and grooming of the intruder. NL3^{R451C} mice, on average, engaged in less non-aggressive interaction with their intruder pairs compared to controls (difference in mean duration = -46 s; p = 0.01; 95% CI: -81,-11; Figure 5C**)**.

Reversal of aggressive phenotype by risperidone treatment

To demonstrate predictive validity in this model, NL3^{R451C} mice were treated with a non-sedative dose (0.05 mg/kg) of risperidone (Supplementary Figure S1), an atypical antipsychotic commonly used to treat aggression in ASD patients[35]. In mutant mice, risperidone treatment reduced the likelihood of the first attack at any point over the 300 s observation period by 6.7 times (Figure 5D, Hazard ratio = 0.15; 95% CI: 0.03, 0.69; p = 0.015). Risperidone treatment of NL3^{R451C} mice also reduced the frequency of attacks on intruder mice compared to saline injected NL3^{R451C} mice (ratio of expected number of attacks = 0.19; p = 0.012; 95% CI: 0.05, 0.69; Figure 5E). Non-aggressive social interaction was increased following risperidone administration in NL3^{R451C} mice (difference in mean duration

= 52 s; p = 0.01; 95% CI: 11, 92; accounting for the effect of multiple test days, Figure 5F). These data establish that a clinically effective drug reduces aggression in this monogenic mouse model of ASD, supporting predictive validity of the NL3 ^{R451C} mouse and utility for preclinical ASD research.

Discussion

We demonstrate that the NL3 R451C mutation is structurally associated with several other clinically identified neuroligin missense mutations, and can cause GI motility dysfunction via an enteric neural mechanism. Our data suggest that GI symptoms may be integral to ASD in patients expressing synaptic mutations and thus provide opportunities for the identification of therapeutic targets.

The expression of *Nlgn3* together with genes coding for other synaptic adhesion molecules in the myenteric plexus indicates that mutations in these genes implicated in ASD may impact ENS function. NL3^{R451C} mice demonstrate altered colonic motility involving enteric $GABA_A$ receptors. These observations identify the GABAergic transmitter system as a potential target for therapeutic intervention in GI disorders and highlight the need for further research into neural control of GI function in ASD.

NL3^{R451C} mice exhibit heightened aggression that is reduced by risperidone; furthermore they show reduced non-aggressive interactions. These findings demonstrate both face and predictive validity of the NL3^{R451C} mouse model of autism and highlight the utility of these mice for behavioral studies. We suggest that evaluation of gastrointestinal function should be included in the battery of tests needed to characterize animal models expressing ASD associated gene mutations. We predict that GI dysfunction is not a trait unique to NL3^{R451C} mice, but will be present in other models of ASD exhibiting synaptic dysfunction. This work

highlights the need for targeted gastroenterological assessments in ASD patients as part of the routine diagnostic procedures.

Evidence that enteric synaptic dysfunction can cause GI problems in ASD patients may lead to novel therapeutic approaches with significant potential benefits for patients[33]. Involvement of GABA in altered gastrointestinal motility is also relevant to the many patients with ASD who are commonly prescribed a range of medications which modulate GABAergic pathways; notably benzodiazepines including Alprazolam, Diazepam and Lorazepam (prescribed to treat anxiety, agitation, nervousness and sleep disorders)[36]. Furthermore, animal studies have demonstrated that Diazepam potentiates GABA effects at the level of single myenteric neurons[37]. Given that a large proportion of ASD patients also exhibit gastrointestinal issues, further investigation and consideration of the effects of GABAergic modulators on gastrointestinal function is needed. Importantly, our observations offer a novel explanation for the mechanisms underlying GI issues in ASD patients.

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Conflict of Interest Statement: The authors declare that no conflict of interest exists.

Supplementary information is available at Molecular Psychiatry's website.

Figure 1

(**A**) Ribbon representation of the structural alignment of human NLGN4-X linked crystal structure in light blue (2xb6.pdb) and human NLGN3 model in gray. The NLGN3 model is based on rat NLGN1 (3vkf.pdb) and was modelled by Swiss-Model[27, 28]. Highlighted in spheres are ASD associated mutation sites. In yellow is the R451C NLGN3 site[6] and red blue and green are the mutation sites reported for NLGN4-X [6, 38, 39]. (**B**) Human NLGN3 in ribbon representation with ASD associated mutations in NLGN3 and NLGN4-X as spheres and predicted protein-protein interface as patches on the surface of NLGN3. (**C**) Close up of the ASD associated mutations closely juxtaposed to R451C (yellow).

Figure 2

Neuroligin (*Nlgn*) 1-3 and neurexin (*Nrxn*) 1 and 2 mRNA is expressed in brain and gut tissue. (**A)** Expression in the adult mouse whole brain (Br) and duodenum (Duo). (**B)** Expression in the myenteric plexus with associated smooth muscle (MP), but little or no expression in mucosa (M) of colon or duodenum. *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) serves as a reference gene. RT-: no reverse transcriptase, +: brain positive control, NTC: no template water control. Gels in A and B were cropped horizontally to improve clarity and conciseness of presentation.

Figure 3

CMMC frequency is depressed by $GABA_A$ antagonists. (**A**) Schematic diagram of a CMMC propagating from oral to anal colon (left panel) and corresponding spatiotemporal map reflecting changes in gut diameter (colored bar) across the length of the colon. Spatiotemporal maps showing CMMC frequency in colonic preparations from WT (**B**) and $NL3^{R451C}$ (**C**) mice under control (con) conditions (**B_1, C_1**) and in the presence of antagonists for $GABA_A$ (bicuculline (bic), 10μM, **B_2, C_2**; gabazine (gab), 10μM, **B_3, C_3**) and $GABA_B$ (CGP 54626 (CGP), 100 nM, **B_4, C_4**) receptors. (**D**) $NL3^{R451C}$ and WT CMMC frequency from a large control dataset ($NL3^{R451C}$ (n = 70) and WT mice (n = 69)) was unchanged. (**E**) Bicuculline treatment reduced the median number of contractions for $NL3^{R451C}$ (n = 16) compared with WT (n = 16) mice. (**F**) Gabazine also reduced the median number of contractions in $NL3^{R451C}$ (n = 11) compared to WT (n = 11) mice. (**G**) CGP had no effect in WT (n = 8) and $NL3^{R451C}$ (n = 9) mice. In (**H-J**), total contractions/15 min are represented as time course data (mean ± s.e.m.); (**H**) bicuculline, (**I**) gabazine, and (**J**) CGP 54626-treated. Box plots represent median interquartile range and range of the data. Vertical and horizontal scale bars in (**A**) represent 5 s, 10 mm and for (**B, C**) in (**B_1**): 2 min, 5 mm respectively. * $p \leq 0.05$, ** $p \leq 0.01$

Figure 4

No change in neuronal proportions immunoreactive for GABA in $NL3^{R451C}$ (n = 3) compared to WT (n = 3) colon. Representative images of myenteric plexus of (**A**) WT and (**B**) $NL3^{R451C}$ distal colon illustrating neurons immunoreactive for Hu (A_1, B_1; red) and GABA (A_2, B_2; green) and merged images (A_3, B_3). Arrows indicate neurons labelled for Hu and GABA. **C**: Bar graph indicates the percentage of Hu-positive neurons immunoreactive for GABA in WT and $NL3^{R451C}$ proximal, mid and distal colonic regions. There is an effect of region ($p<0.0001$). Data shown as mean ± s.e.m.

Figure 5

Increased aggression was observed in $NL3^{R451C}$ mice (n = 6) compared to WT mice (n = 8) in the resident-intruder test over four days of testing. (**A**) $NL3^{R451C}$ mice had a significantly increased probability of initiating a first attack during the 300s observation period. (**B**) $NL3^{R451C}$ mice showed an increase in attack incidence compared to WT mice and spent significantly less time in social interaction compared to WT mice (**C**). (**D**) In $NL3^{R451C}$ mice, risperidone treatment reduced levels of aggression towards intruder mice. (**E**) $NL3^{R451C}$ mice treated with 0.05mg/kg risperidone (n = 5) attacked intruder mice less frequently compared to saline injected $NL3^{R451C}$ mice. (**F**) Risperidone treatment increased non-aggressive social interaction in $NL3^{R451C}$ mice. Values are displayed as median interquartile range and range of data (attack incidence) and mean ± SD (non-aggressive social interactions). * $p \leq 0.05$, ** $p \leq 0.01$.

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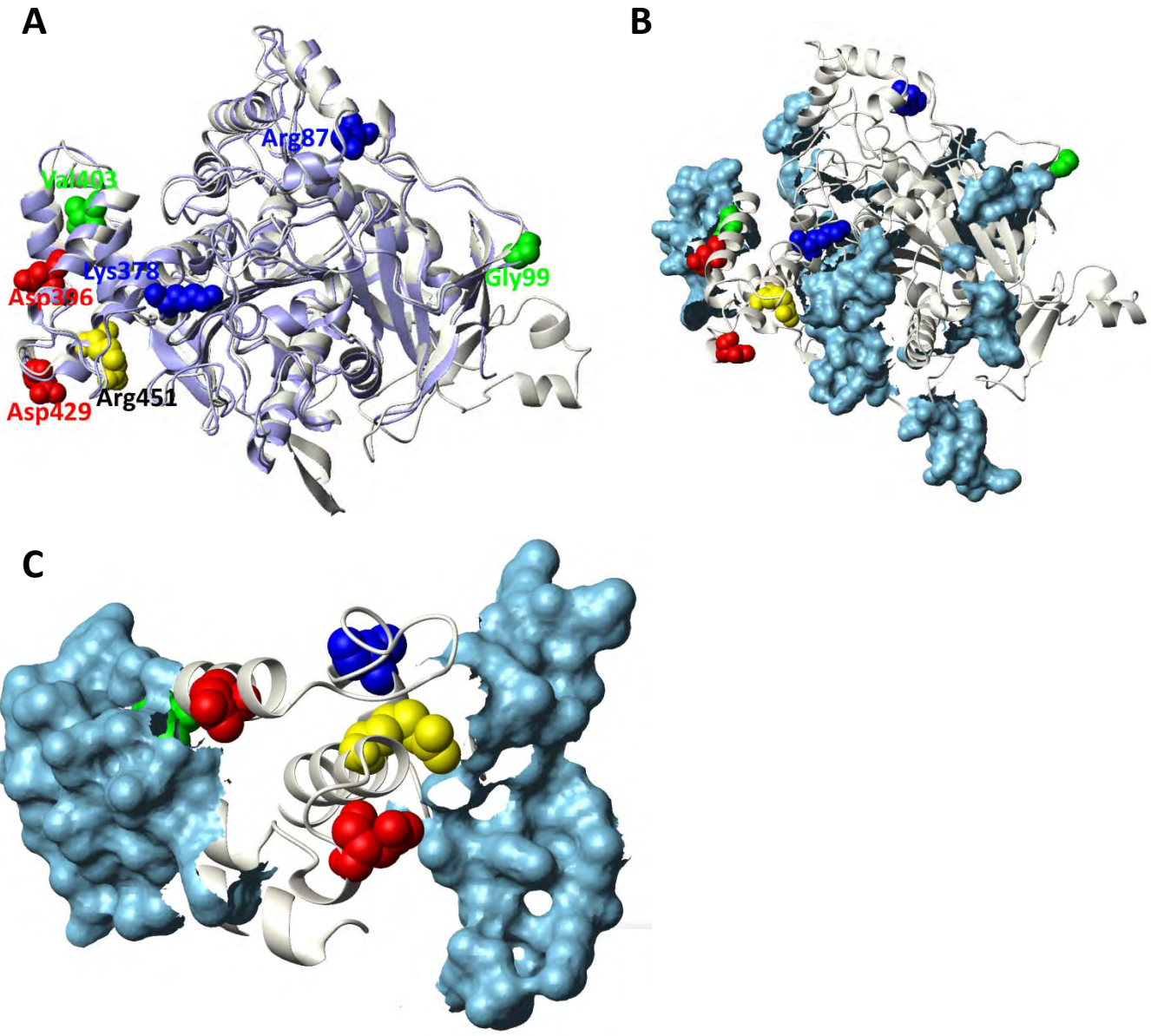
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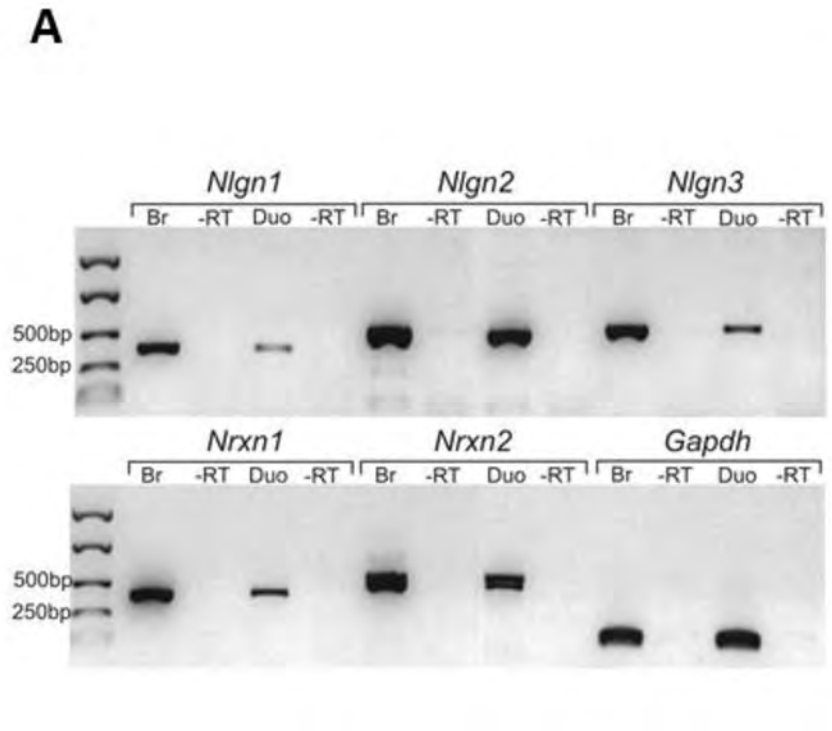
Lys378
Asp396
Gly99
Asp429
Arg451
B
C


A
Nlgn1
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Nlgn3
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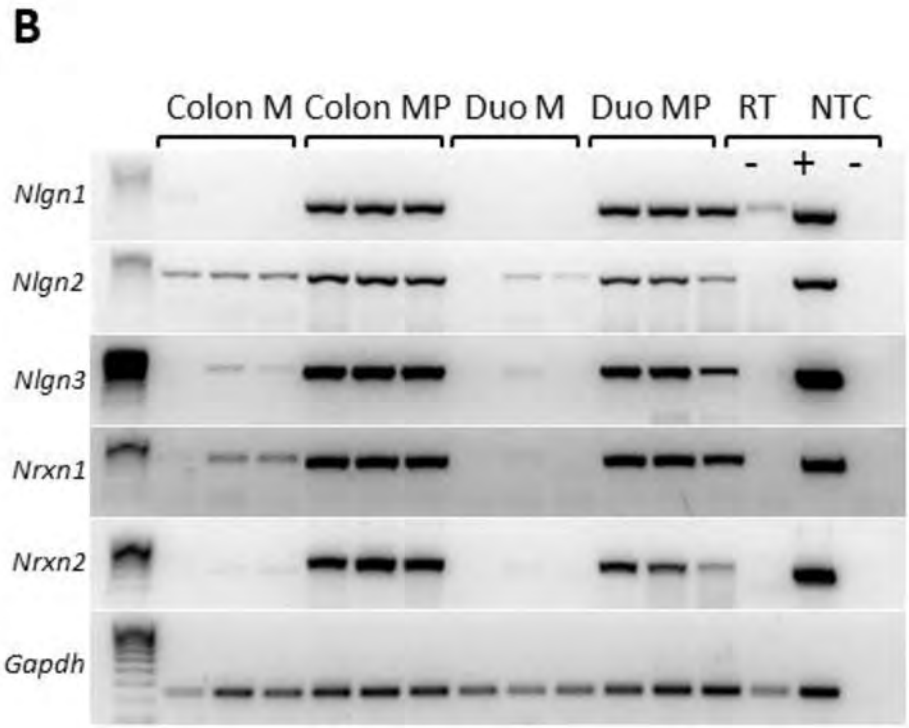
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Colon M
Colon MP
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RT
NTC
- + -
Nlgn1
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Nrxn1
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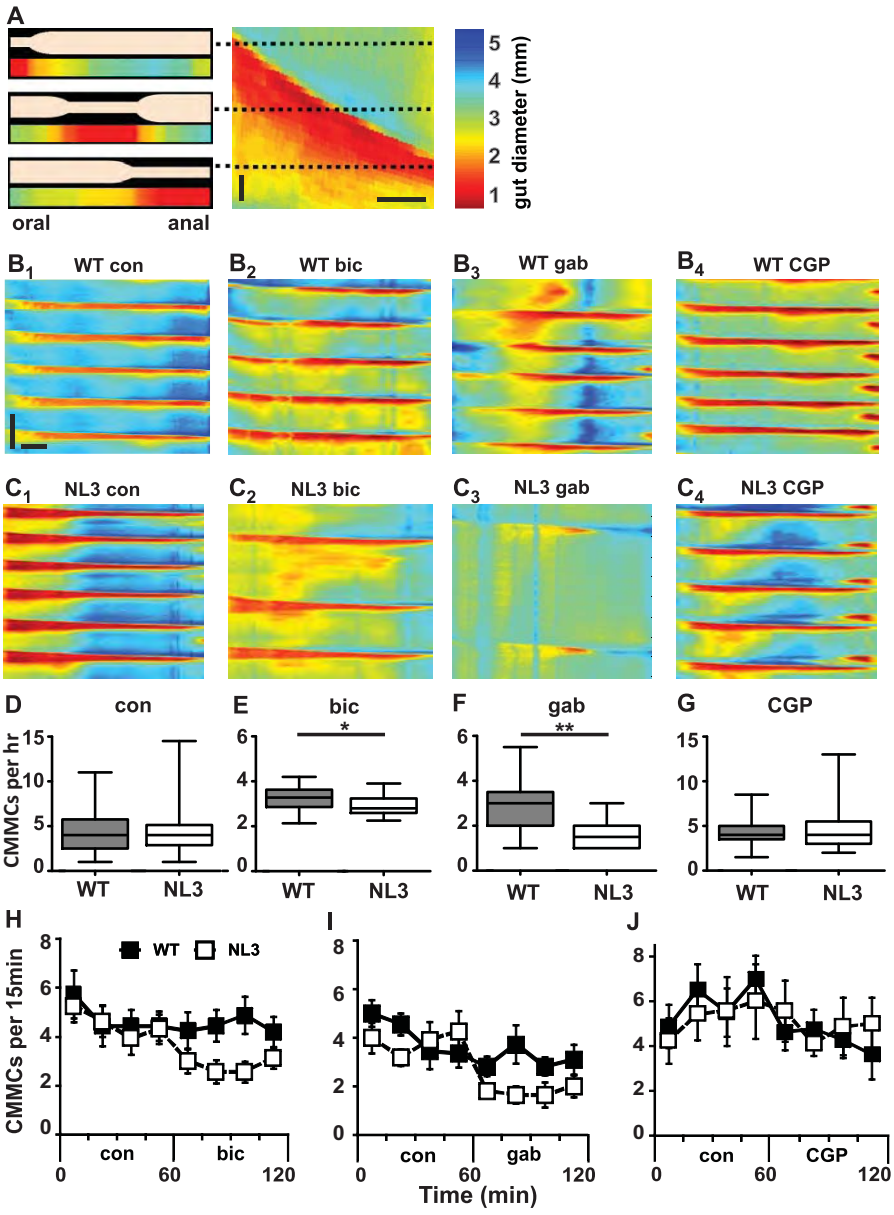
Gapdh


A
oral
anal
5
4
3
2
1
gut diameter (mm)
B1 WT con
B2 WT bic
B3 WT gab
B4 WT CGP
C1 NL3 con
C2 NL3 bic
C3 NL3 gab
C4 NL3 CGP
D con
E bic
F gab
G CGP
CMMCs per hr
WT
NL3
*
**
H
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WT
NL3
CMMCs per 15min
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CGP
Time (min)
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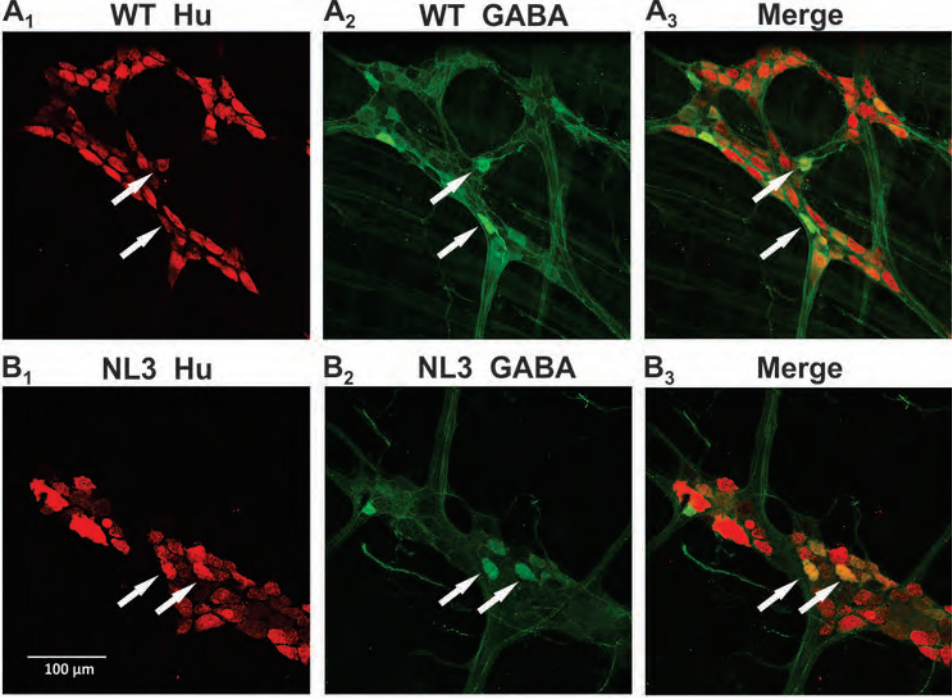
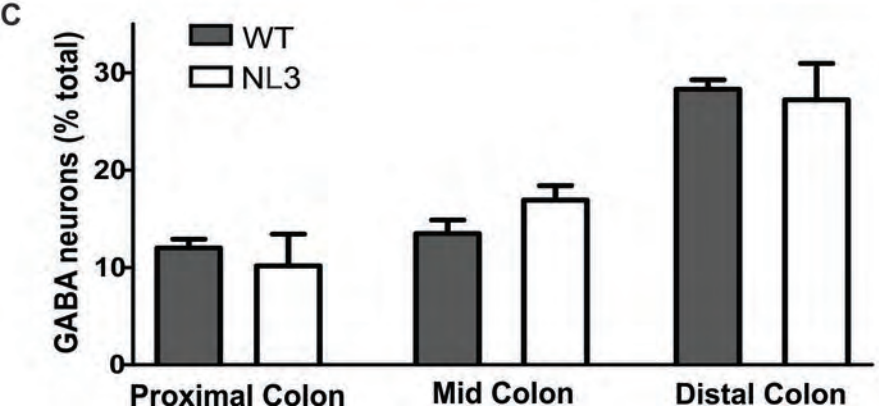
60
120


A1 WT Hu
A2 WT GABA
A3 Merge
B1 NL3 Hu
B2 NL3 GABA
B3 Merge
100 µm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WT
NL3
GABA neurons (% total)
30
20
10
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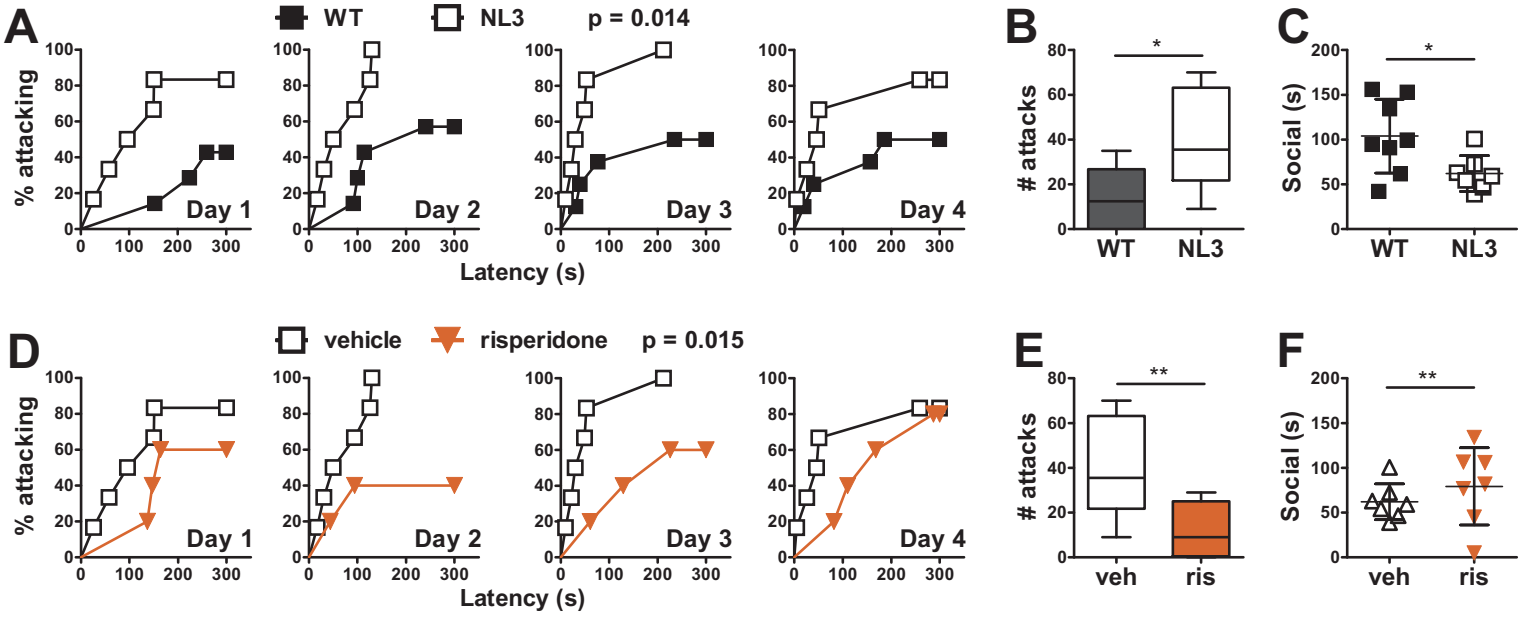
Proximal Colon
Mid Colon
Distal Colon


A
WT
NL3
p = 0.014
% attacking
Day 1
Day 2
Day 3
Day 4
Latency (s)
B
attacks
*
WT
NL3
C
Social (s)
*
WT
NL3
D
vehicle
risperidone
p = 0.015
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Day 1
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Day 3
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Studying autism in rodent models: reconciling endophenotypes with comorbidities

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Autism spectrum disorder (ASD) patients commonly exhibit a variety of comorbid traits including seizures, anxiety, aggressive behavior, gastrointestinal problems, motor deficits, abnormal sensory processing, and sleep disturbances for which the cause is unknown. These features impact negatively on daily life and can exaggerate the effects of the core diagnostic traits (social communication deficits and repetitive behaviors). Studying endophenotypes relevant to both core and comorbid features of ASD in rodent models can provide insight into biological mechanisms underlying these disorders. Here we review the characterization of endophenotypes in a selection of environmental, genetic, and behavioral rodent models of ASD. In addition to exhibiting core ASD-like behaviors, each of these animal models display one or more endophenotypes relevant to comorbid features including altered sensory processing, seizure susceptibility, anxiety-like behavior, and disturbed motor functions, suggesting that these traits are indicators of altered biological pathways in ASD. However, the study of behaviors paralleling comorbid traits in animal models of ASD is an emerging field and further research is needed to assess altered gastrointestinal function, aggression, and disorders of sleep onset across models. Future studies should include investigation of these endophenotypes in order to advance our understanding of the etiology of this complex disorder.

Keywords: autism, epilepsy, sleep, motor deficits, aggression, sensory, gastrointestinal function, anxiety

Studying endophenotypes in rodent models of Autism spectrum disorder (ASD) can offer insights into the heterogeneity and underlying biological causes of this complex disorder. Patients with ASD demonstrate a high degree of variability in both the severity of core diagnostic symptoms (social communication deficits alongside repetitive behaviors) and in the nature and strength of a range of associated comorbidities. If comorbid traits associated with ASD are integral to the disorder we expect that many of these traits will present in animal models. However, despite the prevalence of comorbidities in patients, studies in animal models to date have largely focused on characterizing core behavioral traits. Here we review findings from salient rodent models of ASD identifying endophenotypes that parallel core ASD deficits in combination with one or more comorbid traits commonly reported in patients.

ASD: COMORBID TRAITS

Comorbid traits in ASD include seizures, heightened aggression, and anxiety disorders as well as gastrointestinal problems, altered sensory processing, motor deficits, and sleep disorders (**Table 1**). While treatment of these issues can significantly improve quality of life for patients and their families, the biological mechanisms underlying these symptoms and their co-expression are generally unknown in the context of ASD.

Current estimates for the prevalence of epilepsy in ASD patients range between 8 and 25% (Hara, 2007; Jeste, 2011; Sansa et al., 2011; Woolfenden et al., 2012). Recent meta-analysis data show that epilepsy is more common in ASD patients with an intellectual disability (21.5 vs. 8%; Woolfenden et al., 2012). When epilepsy and abnormal EEG data are compared within the general ASD population, 15% of ASD subjects have an epilepsy diagnosis whereas a larger proportion (24.6%) shows interictal epileptiform EEG abnormalities during sleep (Ekinci et al., 2010). Other reports reveal that as many as 25% of ASD patients have comorbid epilepsy, and that 45.5% show non-seizure-related EEG abnormalities (Parmeggiani et al., 2010). Furthermore, one third (34%) of patients with idiopathic ASD have treatment resistant epilepsy (Sansa et al., 2011).

Aggressive behavior and elevated anxiety are frequently reported in children and adolescents with ASD. Caregiver surveys suggest that as many as 68% of ASD patients show episodes of aggression toward them (Kanne and Mazurek, 2011). Pouw et al. (2013) found that aggression behaviors in ASD are most likely due to a relative impairment in the understanding of the emotions of others. It is also estimated that 40% of ASD patients have at least one anxiety disorder (van Steensel et al., 2011). Specific phobias, obsessive compulsive disorder, and social anxiety disorder are most frequently observed.

A significant proportion of patients with ASD also suffer from gastrointestinal problems (42–90%); with constipation, chronic diarrhea, abnormal stool patterns, and stomach cramps frequently reported (Parracho et al., 2005; Valicenti-McDermott et al., 2006; Ibrahim et al., 2009; Buie et al., 2010; Wang et al., 2011a). Alterations in gastrointestinal function in the context

Table 1 | Clinical comorbidities commonly associated with ASD.

Domain	Comorbid symptoms
Epilepsy	High prevalence of epilepsy (8–25%) and EEG abnormalities (46%) in ASD patients (Amiet et al., 2008; Parmeggiani et al., 2010; Jeste, 2011) High rate of treatment resistant epilepsy in idiopathic autism (34%) (Dudova et al., 2011; Sansa et al., 2011)
Heightened aggression	Approximately 70% of ASD patients exhibit aggression toward caregivers (Kanne and Mazurek, 2011) Reactive aggression correlates with impairments in emotional regulation in children with ASD but not in typically developing children (Pouw et al., 2013)
Anxiety	40% of ASD cases associated with at least one comorbid anxiety disorder (van Steensel et al., 2011)
Gastrointestinal disturbances	Up to 90% ASD patients have chronic GI problems, most commonly constipation, also abdominal pain, diarrhea, and bloating (Parracho et al., 2005; Ibrahim et al., 2009; Buie et al., 2010)
Sensory	Tactile: heightened sensitivity to vibration and thermal pain in palm and forearm (Blakemore et al., 2006; Cascio et al., 2008) Auditory: atypical change detection of auditory stimuli (Gomot et al., 2006; Kwakye et al., 2011) Visual: superior performance in detail oriented tasks, deficits in motion perception (Dakin and Frith, 2005; Latham et al., 2013; Robertson et al., 2013) Altered olfaction and taste in high-functioning ASD patients (Bennetto et al., 2007; Dudova et al., 2011)
Motor impairment	Delays in gross and fine motor domains (Jeste, 2011) Deficits in motor planning, coordination, and gait (Rinehart et al., 2006; Jeste, 2011)
Sleep	Sleep disturbances (quality, quantity, latency to sleep) found in 40–80% of children and adolescents with ASD (Allik et al., 2006; Malow et al., 2006; Jeste, 2011) Sleep onset problems and night waking common in 2- to 5-year-olds with ASD (Krakowiak et al., 2008)

Comorbid traits observed in patients with ASD are heterogeneous and include enhanced seizure susceptibility, heightened aggression, anxiety, gastrointestinal (GI) disturbances, altered sensory and motor function, and sleep disorders.

of ASD are thought to be linked to the effects of anxiety and thereby mediated via CNS function; however investigations into mechanisms involving the enteric nervous system have not been reported.

By far the most common changes associated with ASD are those related to sensory processing which are present in over 90% of individuals diagnosed with ASD (Leekam et al., 2007). Patients with Asperger Syndrome show significantly higher sensitivity to high frequency tactile stimuli compared to control subjects (Cascio et al., 2008). Abnormalities in tactile sensitivity, as well as hypersensitivity to hot and cold stimuli have also been reported in adults with ASD (Blakemore et al., 2006). Auditory processing deficits related to the discrimination of temporally separated tones (Kwakye et al., 2011) and impaired odor detection thresholds (Bennetto et al., 2007; Dudova et al., 2011) have been documented in patients with high-functioning autism as well as subtle impairments in identifying tastes (Bennetto et al., 2007). Interestingly, aberrant motion perception can occur alongside superior visual processing performance in detail oriented tasks, highlighting the potential complexity of sensory changes in ASD patients (reviewed in Dakin and Frith, 2005; also see Latham et al., 2013; Robertson et al., 2013).

Motor abnormalities occur in 60–80% of individuals with ASD and include hypotonia, apraxia, and subtle gait anomalies (see Geschwind, 2009 for review). Abnormal fine and gross motor function, as well as delayed motor learning, dyspraxia, and postural abnormalities are also commonly reported in ASD patients (reviewed in Jeste, 2011). Finally, difficulties initiating sleep, frequent night time waking, and insomnia are frequently reported in children with ASD (Allik et al., 2006; Malow et al., 2006; Krakowiak et al., 2008; Jeste, 2011).

The systematic analysis of traits in animal models corresponding to patient comorbidities can potentially provide insight into the underlying biological mechanisms of ASD. Such outcomes may lead to the design of new therapies and benefits to patients.

ANIMAL MODELS OF ASD

Over the last decade, a substantial number of rodent models of ASD have been generated (reviewed in Silverman et al., 2010a; Peca et al., 2011; Penagarikano et al., 2011; Wang et al., 2011b; Schmeisser et al., 2012; Won et al., 2012) and demonstrate face validity by replicating behavioral traits relevant to ASD. Well-characterized social and communication assessment paradigms and tests for the presence of repetitive behaviors exist for rodent models of ASD (Silverman et al., 2010a). In addition, a battery of tests is available to determine the presence of potential comorbidities including anxiety-like and aggressive behaviors, seizures, disrupted motor activity, sleep dysfunction, and sensory processing deficits (Crawley, 2007) as well as assays for gastrointestinal motility dysfunction (Roberts et al., 2007) in these models. Here we outline findings derived from investigations using these tests (**Table 2**) and highlight areas requiring further research (**Table 3**).

Animal models are discussed in three groups; (i) models with acquired behaviors resulting from environmental insult, (ii) models expressing a human genetic mutation associated with ASD,

Table 2 | Endophenotypes identified in rodent models relevant to comorbid features of ASD.

Domain	Model	Behavior
Seizure susceptibility	VPA	↑ Sensitivity to PTZ (Sobrian and Nandedkar, 1986) and electroshock-induced seizures (Kim et al., 2011)
	PPA	↑ Susceptibility to kindling with repeated intracerebroventricular infusions (MacFabe et al., 2007)
	Shank3B$^{-/-}$	Occasional handling-induced seizures (Peca et al., 2011)
	CNTNAP2	Handling-induced seizures common in adults (Penagarikano et al., 2011)
	FAST	↑ Sensitivity to kindling and chemoconvulsant-induced seizures (McIntyre et al., 1999; Xu et al., 2004; Gilby et al., 2005)
	EL	Handling-induced seizures (Todorova et al., 1999)
	BALB/c	↑ Audiogenic seizures (Morin et al., 1994; Banko et al., 1997)
	C58/J	↑ Sensitivity to PTZ-induced seizures (Nutt and Lister, 1988)
Aggression	Shank2$^{-/-}$	↑ Aggression in home cages although no change in resident-intruder test (Schmeisser et al., 2012)
	FAST	↑ (Reinhart et al., 2004)
	BALB/c	↑ (Brodkin, 2007; Velez et al., 2010)
Anxiety-like behavior	VPA	↑ (Mice) (Markram et al., 2008)
	Shank3B$^{-/-}$	↑ (Peca et al., 2011)
	Shank2$^{-/-}$	↑ (Schmeisser et al., 2012; Won et al., 2012)
	FAST	↑ Fear-potentiated startle (Anisman et al., 2000)
	BALB/c	↑ (Brodkin, 2007)
	BTBR	↑ Under some conditions (McFarlane et al., 2008; Pobbe et al., 2011)
Gastro-intestinal disturbances	BALB/c	Altered intestinal motility compared to C57BL/6 mice in response to serotonin antagonists (Neal et al., 2009)
Sensory	VPA	↓ PPI, ↑ tactile sensitivity (Schneider and Przewlocki, 2005), ↓ olfactory (Schneider and Przewlocki, 2005; Roullet et al., 2010) and pain (Markram et al., 2008) sensitivity
	PPA	↓ Sensorimotor function (increased tendency to slip/fall during beam task; Shultz et al., 2009)
	NL3^{R451C}	↓ Acoustic startle at high decibel levels (Chadman et al., 2008)
	Shank3B$^{-/-}$	↓ PPI (Peca et al., 2011)
	CNTNAP2	↑ Pain and olfactory sensitivity (Penagarikano et al., 2011)
	FAST	↓ Acoustic startle (Anisman et al., 2000)
	BTBR	↓ Thermal response (Silverman et al., 2010b)
Motor	NL3^{R451C}	↑ Latency to fall from rotarod (Chadman et al., 2008)
	Shank3^{e4-9}	Mild motor impairments (Wang et al., 2011b)
	CNTNAP2	Slight ↑ motor coordination (↑ latency to fall from rotarod Penagarikano et al., 2011)
	EL	Delays in visuomotor development (McFadyen-Leussis and Heinrichs, 2005)
Sleep	VPA	Abnormal circadian rhythms (Tsujino et al., 2007)

Endophenotypes relevant to enhanced seizure susceptibility, altered sensory function, and anxiety-like behavior were observed across environmental, monogenetic, and phenotype first models. However, each model was assessed for only a subset of the endophenotypes listed and further research is required to clarify full endophenotypic profiles. VPA, rodents administered valproate.

and (iii) naturally occurring rodent strains that demonstrate behavioral endophenotypes relevant to ASD.

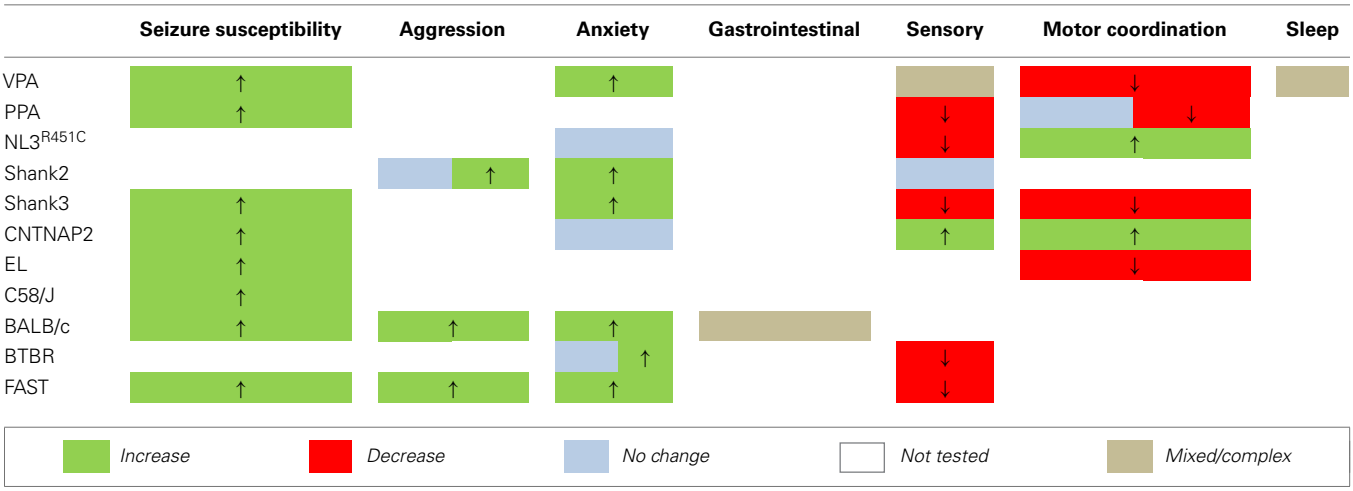
ENVIRONMENTAL MODELS

Autism spectrum disorder-like features exhibited by environmental rodent models are generally elicited in response to an overt insult or developmental challenge, such as exposure to toxins resulting in altered neurological development.

Valproate models

During pregnancy, maternal exposure to the first generation antiepileptic drug valproate has been shown to significantly increase the risk of ASD in children (Rasalam et al., 2005; Meador et al., 2006; Bromley et al., 2008). Valproate is a short-chain fatty acid and is thought to reduce neuronal excitability primarily by increasing concentrations of the inhibitory neurotransmitter GABA and modulating voltage-gated sodium channels (Chapman et al., 1982; Rogawski and Loscher, 2004). In both mice and rats, exposure to valproate during gestation via intraperitoneal injection or orally with food produces deficits in social interaction and repetitive behaviors (Schneider and Przewlocki, 2005; Wagner et al., 2006; Roullet et al., 2010; Kim et al., 2011). These animals also show reduced sensitivity to pain (Markram et al., 2008) and olfactory cues (Schneider and

Table 3 | An overview of endophenotypes assayed in rodent models of ASD.



Seizure susceptibility, sensory function, motor coordination, and anxiety-like behaviors are most commonly tested across models. Aggressive behavior, gastrointestinal function, and sleep cycles are generally understudied. Dual colored cells: formal aggression testing in Shank2$^{-/-}$ mice did not yield data suggesting abnormal aggressive behavior, however excessive aggression was observed in home cages; PPA rats had impaired sensorimotor abilities when tested using the beam task but showed no change in swim speed in other assays. VPA, rodents administered valproate.

Przewlocki, 2005; Roullet et al., 2010), increased tactile sensitivity (Schneider and Przewlocki, 2005), and diminished acoustic pre-pulse inhibition, a test commonly used to index abnormalities in sensorimotor gating (Schneider and Przewlocki, 2005; Markram et al., 2008; Gandal et al., 2010; Roullet et al., 2010). Valproate-exposed adult rats show increased levels of anxiety-like behaviors (Markram et al., 2008) and a reduced threshold for electroshock (Kim et al., 2011) and pentylenetetrazole (PTZ)-induced seizures (Sobrian and Nandedkar, 1986). These rats also show altered circadian rhythms characterized by frequent arousal during the light/sleep phase (Tsujino et al., 2007; **Tables 2** and **3**).

Propionic acid model

The gut microbiota have been suggested to play a role in the etiology of ASD (Mulle et al., 2013). Potential mechanisms contributing to ASD phenotypes are unknown, however excess toxin-producing bacteria have been identified in patients with ASD (Parracho et al., 2005) and increased levels of short-chain fatty acids (such as propionic acid; PPA) produced by enteric bacteria have been studied in rats (MacFabe et al., 2007). In rodent models, administration of the endogenous short-chain fatty acids butyric acid (Thomas et al., 2010), sodium acetate (Shultz et al., 2008, 2009), and PPA directly into the cerebral ventricles produces endophenotypes relevant to ASD (MacFabe et al., 2007, 2011; Shultz et al., 2008, 2009; Thomas et al., 2010). Acute intracerebral ventricular infusion of PPA in rats reduces sociability and learning and also produces sensorimotor impairments (Shultz et al., 2009). This paradigm also results in reduced cognitive flexibility during reversal learning (MacFabe et al., 2011). Furthermore, repeated intraventricular PPA infusion leads to increased susceptibility to kindling-induced seizures and stereotypic behavior (MacFabe et al., 2007, 2011; Shultz et al., 2009; **Tables 2** and **3**).

A small number of ASD patients (5%) show mitochondrial dysfunction along with altered levels of various metabolites suggestive of altered fatty acid processing (Frye et al., 2013). Further investigation to assess the effects of both PPA and valproate on gastrointestinal function (i.e., following oral administration) is needed (see **Table 3**), as the short-chain fatty acid receptor (GPR43) expressed by some mucosal enteroendocrine cells may play a role (Karaki et al., 2006). The effects of orally administered PPA in particular would be of interest and would serve to strengthen construct validity of this model.

GENETIC MODELS

Many gene mutations associated with ASD code for proteins involved in the formation and maintenance of synapses (Sudhof, 2008; Betancur et al., 2009; Bourgeron, 2009; Chakrabarti et al., 2009; Betancur, 2011; Geschwind, 2011). Here we review findings from monogenic mouse models expressing mutations in four genes modulating synaptic function; the neuroligin-3^{R451C} (NL3^{R451C}) mice (Tabuchi et al., 2007; Chadman et al., 2008) two models expressing specific mutations in the Shank3B/ProSAP2 gene [Shank3B knockout mice and Shank3B^{e4-9} partial knockout mice (Peca et al., 2011; Wang et al., 2011b)], as well as two SHANK2 knockout models (Schmeisser et al., 2012; Won et al., 2012) and the contactin associated protein-like 2/Neurexin IV (CNTNAP2/NRXN4; Penagarikano et al., 2011) knockout mouse model (**Table 2**). Electrophysiological studies in these mice report altered glutamatergic and GABAergic synaptic function (Tabuchi et al., 2007; Etherton et al., 2009, 2011; Peca et al., 2011; Wang et al., 2011b; Schmeisser et al., 2012; Won et al., 2012). Each of these models also expresses strong ASD behavioral endophenotypes suggesting a role for these genes in shaping core behaviors relevant to ASD diagnosis. However, it is not well established whether these animal models replicate comorbid traits observed in patients.

Neuroligin-3^{R451C} mice

Neuroligins are adhesion molecules which interact with a range of post-synaptic scaffolding proteins including Shank3 and CNTNAP2 and bind to members of the presynaptic neurexin family across the synaptic cleft (Sudhof, 2008; Krueger et al., 2012; Verpelli and Sala, 2012). Mutations in the neuroligin family of post-synaptic adhesion molecules were implicated in ASD after a spontaneous point mutation in the gene encoding NL3 was identified in two brothers with ASD; one with comorbid epilepsy (Jamain et al., 2003). Mice expressing the NL3^{R451C} mutation show a subtle reduction in pup distress calls (on post-natal day 8) and reduced acoustic startle (Chadman et al., 2008). Under some conditions and on some genetic backgrounds, NL3^{R451C} mice also show impaired social interaction (Tabuchi et al., 2007; Etherton et al., 2011). Delays in meeting developmental milestones (e.g., slower righting reflexes), which may appear as motor deficits early in development, have also been observed in these mice (Chadman et al., 2008). However, adult NL3^{R451C} mice showed better motor coordination in the accelerating rotarod test compared with wild type littermates (Chadman et al., 2008).

Shank3-related models

The Shank (SH3 and multiple ankyrin repeat domains) gene family (also known as Proline-rich synapse-associated proteins; ProSAPs) contains three members; Shank1-3 that code for post-synaptic scaffolding proteins involved in the recruitment of several receptors and proteins (including the neuroligins and neurexins) to the excitatory post-synaptic membrane (Irie et al., 1997; Meyer et al., 2004; Baron et al., 2006; Hayashi et al., 2009; Arons et al., 2012). Rare microdeletions within the 22q13 locus (containing Shank3) are associated with intellectual disability, speech delay, and ASD (Nesslinger et al., 1994; Bonaglia et al., 2006; Durand et al., 2007). Mutations in Shank2 are also associated with ASD (Berkel et al., 2010; Kumar, 2010). Two different genetic models in which Shank3 is altered; Shank3B$^{-/-}$ (Peca et al., 2011) and Shank3^{e4-9} (Wang et al., 2011b) in addition to two recently reported Shank2 knockout models (Schmeisser et al., 2012; Won et al., 2012) demonstrate core and comorbid traits relevant to ASD. A third model in which one full length copy of Shank3 is deleted shows core ASD endophenotypes; however the expression of secondary/comorbid features outlined here has not been investigated in these mice (Bozdagi et al., 2010). Shank3B$^{-/-}$ mice lacking the Shank3α and β isoforms show increased repetitive behavior (self-injurious grooming) and reduced interaction with a stranger mouse as well as occasional handling-induced seizures (Peca et al., 2011 and reviewed in Herbert, 2011). Shank3^{e4-9} mice (in which exons 4–9 are deleted) show core ASD-like deficits including social impairments, repetitive behaviors, and altered communication (i.e., less complex vocalization patterns), with learning deficits and mild motor abnormalities also evident (Wang et al., 2011b). In addition to a role as a structural protein in the central nervous system, Shank3 is present at enteric nervous system synapses (Huett et al., 2009). The enteric nervous system controls gastrointestinal motility and mucous secretion and therefore gene mutations leading to changes in synaptic function (including many ASD candidate genes) may also affect gastrointestinal function (Gershon and Ratcliffe, 2004). The Shank3 mouse models of ASD are therefore excellent candidates for investigating effects of ASD-associated gene mutations on gastrointestinal motility. Shank2 knockout mice demonstrate abnormal vocal and social behaviors, and increased grooming behaviors. Hyperactivity (e.g., repetitive jumping) and anxiety-like behaviors have also been reported in these mice (Schmeisser et al., 2012; Won et al., 2012). Schmeisser et al. (2012) detected no change in aggressive behaviors in Shank2 knockout mice using a resident-intruder assay. Despite this negative result, a high level of aggression between Shank2 knockout males was observed in home cages (Schmeisser et al., 2012).

CNTNAP2 mice

Genetic ablation of the contactin associated protein-like 2 (CNTNAP2) gene, a member of the neurexin transmembrane protein superfamily (also known as CASPR2 and Neurexin IV), results in ASD-like deficits in social interaction and stereotypic behaviors in mice (Penagarikano et al., 2011). In addition, CNTNAP2 knockout mice show hyperactivity, impaired nest building, and frequent handling-induced seizures after 6 months of age (Penagarikano et al., 2011). The CNTNAP2 gene has been associated with ASD and a recessive form of epilepsy (Strauss et al., 2006). These mice exhibit sensory endophenotypes including hyper-reactivity to thermal sensory stimuli and superior performance in the buried food test, an assay for olfactory function (Penagarikano et al., 2011). CNTNAP2 knockout mice also showed slightly improved motor coordination on the rotarod compared to wild type littermates. Perhaps surprisingly, the atypical antipsychotic risperidone (prescribed to treat aggression and irritability in some cases of ASD) reversed nest building deficits as well as locomotor hyperactivity in these mice (Penagarikano et al., 2011), demonstrating predictive validity in this model (**Table 2**).

Behavioral analyses in transgenic mouse models of ASD confirm that a range of proteins regulating synaptic function are likely to be integral to this disorder. Most studies involving genetic models have investigated one or two endophenotypes relevant to patient core and comorbid traits (**Tables 2** and **3**). However, to better understand the relationship between these traits a focus on assessing the more subtle secondary endophenotypes is required. Seizure susceptibility, gastrointestinal function, sleep cycles, and aggressive behaviors remain to be investigated in the majority of these genetic models of ASD (**Table 3**). Still, the presence of endophenotypes relevant to comorbid traits of ASD in each of these genetic models suggests that at least some of these traits may be associated with the core behavioral features of the disorder.

PHENOTYPE FIRST MODELS

Interplay between genomic and non-genomic influences (e.g., maternal effects) is almost certainly involved in the symptom heterogeneity associated with ASD. To further understand their relative degree of contribution, animal models in which clinically relevant endophenotypes occur "naturally" are of great interest. There are currently several rodent models developed via breeding processes alone that exhibit measurable endophenotypes relevant to the diagnostic criteria and comorbid traits associated with ASD. These animal models include the FAST/SLOW rats and the C58/J, BALB/c, BtBR T + tf/J (BTBR), and epileptic-like (EL) mice (**Tables 2** and **3**).

FAST/SLOW rats and EL mice

The FAST and SLOW rat strains were derived from parent populations of Long Evans Hooded and Wistar rats using selective breeding processes based on relative seizure susceptibility in the amygdala kindling model (Racine et al., 1999). This process ultimately produced a seizure-prone (FAST) and seizure-resistant (SLOW) strain. FAST rats have since proven highly seizure-prone in both the kindling model and in chemoconvulsant (e.g., pilocarpine, kainate) seizure-induction models (McIntyre et al., 1999; Xu et al., 2004; Gilby et al., 2007; Gilby and O'Brien, 2013). EL mice, like FAST rats, were also created via selective breeding based on relative seizure susceptibility and originated from the non-epileptic DDY mouse strain (Meidenbauer et al., 2011). EL mice typically exhibit handling-induced seizures by postnatal day 50–60 (Todorova et al., 1999). Remarkably, the breeding processes used to create heightened seizure sensitivity in both colonies simultaneously produced robust, comorbid ASD-like traits. Both FAST rats and EL mice exhibit significant social impairment (Reinhart et al., 2004, 2006; Gilby et al., 2007; Lim et al., 2007; Turner et al., 2007) and repetitive behaviors (e.g., overgrooming, self-injurious scratching, and/or myoclonic jumping; Gilby, 2008; Meidenbauer et al., 2011) alongside delays in social, physical, and visuomotor development (McFadyen-Leussis and Heinrichs, 2005), learning deficits, impulsivity, and hyperactivity in various testing paradigms (Anisman and McIntyre, 2002; McFadyen-Leussis and Heinrichs, 2005; Azarbar et al., 2010). FAST rats are also more aggressive than their comparison (SLOW) strain (Reinhart et al., 2004, 2006) and show reduced acoustic startle but enhanced fear conditioning (Anisman et al., 2000). Thus, FAST rats and EL mice offer a similar endophenotypic profile relevant to core and comorbid symptoms observed in ASD.

C58/J mice

C58/J mice naturally exhibit ASD-like traits including poor sociability (Moy et al., 2008; Ryan et al., 2010), relative learning deficits, hyperactivity (Moy et al., 2008), and stereotypic behaviors (i.e., jumping and flipping; Ryan et al., 2010). Interestingly, C58/J mice also demonstrate a reduced threshold for PTZ-induced seizures (Nutt and Lister, 1988). However, in contrast to the ASD-like developmental delays observed in FAST and EL animals, C58/J mice meet developmental milestones earlier than their comparison strain (C57BL/6J; Ryan et al., 2010).

BALB/c and BTBR mice

The BALB/c and BTBR mouse strains exhibit core ASD traits in the form of impaired social interaction and repetitive behaviors (i.e., overgrooming and/or excessive marble burying; Brodkin, 2007; Shoji and Kato, 2009; Pearson et al., 2011). BTBR mice also demonstrate increased social anxiety-like behavior (Pobbe et al., 2011) although anxiety responses to novel situations are inconsistent (McFarlane et al., 2008). BTBR mice are less reactive to thermal (hotplate) stimuli than the C57Bl/6J standard strain (Silverman et al., 2010b), suggesting subtle sensory changes exist in this model. In addition, several BALB/c substrains displaying distinct behavioral phenotypes offer particular strengths for comorbidity investigation. BALB/cJ mice exhibit altered gastrointestinal function (Neal et al., 2009) and are highly aggressive (Velez et al., 2010) while the epilepsy-prone (EP) BALB/c substrain is susceptible to audiogenic seizures (Morin et al., 1994; Banko et al., 1997). Notably, BTBR and BALB models have a high incidence of corpus callosal agenesis and severely reduced hippocampal commissural volumes (Wahlsten et al., 2003), which may be relevant to reports of reduced corpus callosal volumes in ASD patients (Anderson et al., 2011).

The characterization of ASD-relevant traits in these "natural" models is a relatively new initiative. Still, the documented commonalities thus far are striking; particularly the co-expression of repetitive behaviors and impaired social interaction together with heightened seizure sensitivity (**Table 3**). Finally, while we are aware that a few studies have investigated aggression and sensory processing in these rodent models, further testing using validated assays (Silverman et al., 2010a) should be applied to fully characterize the presence of core and comorbid features in these models.

SUMMARY

The primary aim of this review was to compare endophenotypic clustering within a selection of animal models of ASD. Here we focus on models expressing at least two core ASD endophenotypes with additional endophenotypes relevant to comorbid traits reported in ASD patients.

ENDOPHENOTYPING: A NEW APPROACH

We report that models generated via environmental insult, genetic manipulation, and selective breeding processes demonstrate a number of overlapping endophenotypes (**Tables 2** and **3**) relevant to both clinical comorbid (**Table 1**) and core traits of ASD. Detailed investigation into the more subtle endophenotypes associated with these models is a relatively novel approach. Indeed, many clinical traits highlighted here have yet to be investigated in these models or should be re-examined using consistent methodological approaches. Until then any ranking of the clinical relevance of the phenotypic profiles would be premature. Interestingly however, enhanced seizure susceptibility, altered sensory function, anxiety-like behaviors, and changes in motor coordination were the most frequently reported endophenotypes across models (**Table 3**). Although not routinely investigated, several of the models also showed atypical aggressive interactions (**Tables 2** and **3**). Despite evidence for disturbed sleep and abnormal gastrointestinal function in a significant number of ASD patients (see **Table 1**), to our knowledge, circadian rhythms and gastrointestinal function have only been investigated in two models; valproate-exposed rats and BALB/c mice, respectively. As discussed, gastrointestinal motility was insensitive to serotonin antagonists in BALB/c mice in comparison to a control strain (Neal et al., 2009) and valproate-treated rats showed increased arousal during sleep compared to untreated controls (Tsujino et al., 2007; **Tables 2** and **3**).

OVERLAPPING TRAITS

The presence of both core and comorbid endophenotypes in a range of animal models suggests that at least some of these traits may be interrelated and possibly integral to the etiology of ASD. Some endophenotypes are indeed co-expressed across different model constructs (for example, seizure susceptibility is consistently increased, as are anxiety-like behaviors in examples of environmental, genetic, as well as phenotype first models;

Table 3). Both environmental models (i.e., rodents administered the fatty acids valproate and PPA) and phenotype first models show heightened seizure susceptibility and anxiety-like behaviors together with sensory and motor deficits (**Table 3**). In contrast, genetic models show varied changes in sensory and motor domains (**Table 3**) for which the underlying mechanisms are unknown.

FUTURE DIRECTIONS: POTENTIAL MECHANISMS UNDERLYING ASD ENDOPHENOTYPES

Animal models are an important tool with which to tease apart the biological mechanisms underlying ASD. Given the diverse nature of ASD, it is unlikely that a single cause is responsible for this disorder and more recent research suggests some degree of interaction between the CNS and peripheral systems. Many gene mutations identified in patients with ASD affect synaptic function (Betancur et al., 2009; Bourgeron, 2009; Betancur, 2011). This supports an emerging hypothesis that ASD is primarily a disorder of neuronal communication (Grabrucker et al., 2011; Ebert and Greenberg, 2013) and we suggest that subtle changes in neural function could underlie many of the comorbid traits described here. For example, it is well established that gene mutations coding for ion channels that result in altered synaptic function in the CNS can cause seizures in patients (Helbig et al., 2008; Goldberg and Coulter, 2013). It is also important to acknowledge, however, that many neurotransmitters and receptors that regulate neuronal communication in the CNS are of functional importance in the periphery and may thereby contribute to common comorbid traits in patient subsets. For example, in the case of gastrointestinal issues, many of the synaptic genes associated with ASD including the Shanks, neurexins, and neuroligins are also expressed in the enteric nervous system (Huett et al., 2009; Raab et al., 2010; Zhang et al., 2013), which regulates gastrointestinal motility and secretion. It is, therefore, feasible that synaptic mutations may underlie gastrointestinal symptoms in at least a subset of patients with ASD (Gershon and Ratcliffe, 2004) in addition to altering neuronal communication in the CNS. Future research should explore potential neural mechanisms underlying endophenotypes, in particular, those that are currently understudied (such as gastrointestinal disorders and altered circadian rhythms) in animal models of ASD.

In summary, rigorous endophenotyping in animal models of ASD can assist in identifying the molecular mechanisms underlying these common comorbid traits. Such information may also contribute to the identification of putative patient subsets within this spectrum of disorders and the subsequent tailoring of potential therapies. However, in order to achieve these goals, a more consistent approach in the assessment and comparison of endophenotypes is needed.

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COMMENTARY

Translating Preclinical Environmental Enrichment Studies for the Treatment of Autism and Other Brain Disorders: Comment on Woo and Leon (2013)

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Environmental enrichment (EE) has been shown to induce beneficial effects in mouse models of autism spectrum disorder (ASD), as well as animal models of a variety of other neurological and psychiatric disorders. Investigation of the mechanisms responsible for these changes in animal models will facilitate translation of EE and associated therapies to patient cohorts. In the accompanying article, Woo and Leon demonstrate clinical benefits of sensorimotor enrichment in patients with ASD. We discuss the implications of these findings for future development of therapeutic approaches for ASD and other brain disorders.

Keywords: environmental enrichment, Autism Spectrum Disorder, sensory, animal models, treatment

Woo and Leon's (2013, pp. 487–497) study applies environmental enrichment (EE) in the form of increased sensorimotor stimulation to patients and identifies significant improvements in subjects exposed to EE compared with controls. This work is important, as it attempts to directly translate work from animal models to benefit patients with autism spectrum disorder (ASD). The fact that this study shows improvements in cognitive performance and autism severity following sensory enrichment in this relatively small cohort is promising. Following appropriate follow-up on a larger scale, this EE approach for ASD could be developed for widespread applications. The effective treatment of behavioral aspects of ASD with sensorimotor enrichment holds several lifestyle advantages for individual patients, in addition to socioeconomic benefits to general communities.

Sensory abnormalities are present in the overwhelming majority of ASD patients (Leekam, Nieto, Libby, Wing, & Gould, 2007) and pose significant challenges for these patients and their families (Schaaf, Toth-Cohen, Johnson, Outten, & Benevides, 2011). Such abnormalities may present as apparent indifference to pain/heat/cold, adverse response to specific sounds or textures, excessive smelling or touching of objects, and/or fascination with lights or spinning objects. The fact that sensory processing abnormalities are a prominent feature of ASD further reinforces the need for treatment of these symptoms in patients and the potential for impacting other symptoms of ASD by manipulating neural pathways involved in sensory processing. Sensory alterations are emerging as an integral component of ASD, and modulation of sensory processing may assist in improving other core behavioral features of ASD, for example, stereotypy (Sidener, Carr, & Firth, 2005).

In light of the high prevalence and impact of inappropriate sensory processing in ASD, the implementation of EE paradigms to benefit ASD patients is a logical and positive step. However, there are a number of issues to consider in order for such an approach to be optimized for wider clinical implementation. Patient heterogeneity predicts that individuals with ASD will respond to EE in differing ways. These responses may be due to complex genetics and environmental influences resulting in differing levels of sensory abnormalities or responsiveness of people with ASD at baseline.

The study by Woo and Leon (2013) identifies a potential therapy that has several practical advantages for patients and their families. The implementation of an EE protocol that can be conducted by parents (following minimal training) in the home using inexpensive materials has broad applications. For the community, it is clear that there are substantial socioeconomic advantages to undertaking this kind of approach. For families and individuals, timely access to services (including diagnostic evaluation) is a major issue, and perhaps if these treatments are targeted to patients for whom this will be effective, accessibility to other services in general can be improved. The benefits to patients (who, together with sensory issues, often exhibit anxiety disorders) and families in having the ability to carry

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out the treatment within the home cannot be overstated. The minimal likelihood of unwanted or harmful side effects associated with this treatment is another advantage of this approach.

Benefits of EE Protocols: What Are the Essential Components?

It may be that close examination of the essential components of the EE paradigms in both animal models and patients can be conducted in order to achieve maximal improvements across laboratories and in the clinical setting. To date, studies focused on one or two behavioral outcomes in response to tailored enrichment programs have shown improvement in some symptoms of ASD. For example, the reduction of stereotypy (Sidener et al., 2005), or stereotypy in addition to reduced sleep problems and off-task behavior (Escalona, Field, Singer-Strunck, Cullen, & Hartshorn, 2001), have previously been demonstrated in response to tailored (predominantly tactile) enrichment programs.

EE in Animal Models of ASD

Standard housing of rodents in the laboratory setting is comparable with a state of sensory deprivation, and this has implications for the potential translation of preclinical studies (Nithianantharajah & Hannan, 2006). Wild-type laboratory animals subjected to EE protocols exhibit enhanced neural plasticity, such as increased adult neurogenesis, dendritic branching, synaptic numbers, and other measures of synaptic plasticity, such as LTP, which are associated with cognitive enhancement (Nithianantharajah & Hannan, 2006; van Praag, Kempermann, & Gage, 2000). Furthermore, beneficial impacts of EE in animal models of disease have also been demonstrated at molecular, cellular, behavioral, and cognitive levels (Laviola, Hannan, Macri, Solinas, & Jaber, 2008; Pang & Hannan, 2013). Such benefits are also evident in mouse models of syndromic ASD, such as Fragile X syndrome (Restivo et al., 2005) and Rett syndrome (Kondo et al., 2008). A recent report also demonstrated that EE is effective in a mouse model of Potocki-Lupski syndrome exhibiting copy number variation (CNV; Lacaria, Spencer, Gu, Paylor, & Lupski, 2012). Specifically, motor, social, and cognitive enrichment ameliorated motor defects, improved learning and memory defects, reduced aggressive behavior, and relieved anxiety in these mice.

In animal models of nonsyndromic ASD, the effects of EE remain largely unexplored. This is not due to a lack of animal models. At least a dozen mouse models of ASD have been generated based on monogenic mutations identified in patients (Etherton, Blaiss, Powell, & Sudhof, 2009; Peça et al., 2011; Peñagarikano et al., 2011; reviewed by Silverman, Yang, Lord, & Crawley, 2010). Other rodent models show ASD-relevant behaviors following environmental insult, such as exposure to the antiepileptic drug valproate during gestation (Roullet, Wollaston, Decatanzaro, & Foster, 2010; Schneider & Przewlocki, 2005). A third group of animal models of ASD are standard inbred laboratory strains (Moy et al., 2008) or strains derived via selective breeding (Meidenbauer, Mantis, & Seyfried, 2011; Racine, Steingart, & McIntyre, 1999) that show behaviors characteristic of ASD. Increased repetitive behavior and reduced social interaction and communication are common in these models, but many other traits remain to be carefully investigated before the effects of EE on behaviors that parallel the broader range of ASD symptoms (e.g., sensorimotor function, maladaptive behavior, and cognitive function) can be assessed.

Sensorimotor function in rodent models is assessed using a range of standard assays (Crawley, 2007), and data are now emerging for some of these animal models of ASD. Although paradigms for hypo- and hypersensory responses are not routinely included when testing rodent models of ASD, when examined, sensory changes are widely observed across these models. Altered responses to olfactory stimuli are seen in the CNTNAP2 genetic model of ASD (Peñagarikano et al., 2011) as well as in valproate rodent models (Roullet et al., 2010; Schneider & Przewlocki, 2005). Differences in tactile response (Schneider & Przewlocki, 2005), thermal response (Silverman, Yang, Turner, et al., 2010), acoustic startle (Anisman et al., 2000; Etherton et al., 2009), as well as changes in measures of prepulse inhibition (PPI; Etherton et al., 2009; Peça et al., 2011; Schneider & Przewlocki, 2005) are also evident across these models. The fact that each of the models described here show behaviors relevant to core features of ASD in patients in addition to sensory alterations suggests strong face validity for these models and utility in preclinical investigations. The next step will be to expose these models to EE and investigate whether both core ASD endophenotypes and other relevant behavioral traits are rescued.

If treatment with EE is able to rescue relevant phenotypes in additional rodent models of ASD, this will help identify underlying biological mechanisms in these models. For example, in the case of monogenic models of autism expressing a range of gene mutations that alter synaptic function, positive responses to EE in terms of reducing endophenotypes, or even rescue of core phenotypes, may provide possibilities for clinical translation. Furthermore, research in models can then examine molecular mechanisms mediating therapeutic EE responses. Such data may also provide clues to identifying patient subsets likely to respond to sensorimotor enrichment as a treatment for ASD core and comorbid traits. Further characterization of sensorimotor responses in these models will lay the ground work for testing the effects of various components of EE across temporal windows and identifying the mechanisms involved.

ASD encompasses a wide range of heterogeneous symptoms, and the precise developmental trajectories of various subsets of patients are not known. We emphasize that the approach used in this study by Woo and Leon (2013) demonstrates strong potential for clinical translation. Nevertheless, in order to identify the most efficient paradigms and ensure that therapies are tailored to individual patients, we also highlight remaining shortcomings, which could be addressed in future larger scale randomized controlled trials.

Future Directions for Translational Research in ASD

A critical issue for the broader field of ASD research regards the heterogeneity of this disorder and correlation with treatment outcomes. In a larger study, it would be advantageous to include detailed patient profiles prior to EE. Data describing the degree and presence or absence of comorbid traits (e.g., epilepsy, sleep disorders, motor deficits, anxiety levels) expressed by subjects may provide clues to identifying those patients most likely to benefit from sensorimotor enrichment. Any measures that contribute to predicting patient response to treatment (EE and others) have the potential to decrease health expenditure and increase the availability of relevant services to patients.

A larger trial may also assist in identifying the more effective components of the therapy by scrutinizing the consistency between subject protocols and materials used in the EE. The authors note

that olfactory stimuli together with tactile stimulation created a lasting recognition of scents in human newborns in contrast with exposure to scent alone (Sullivan et al., 1991). Would the assessment of a group subjected to olfactory stimulation together with tactile sensory enrichment in the absence of other components of the enrichment paradigm result in a similar positive outcome? Any such protocol refinement could reduce the time and resources required, thus increasing the potential for widespread clinical implementation.

When considering translation to patients, it is important to determine the most effective components of EE treatment. This is a complex problem, as it is likely that subsets of patients will show a range of responses to similar stimuli. Clarification of these questions will ensure that time and resources are best managed with respect to patient exposure to enrichment paradigms. Perhaps as a starting point, testing a larger cohort of patients for responses to sensory enrichment would be worthwhile and inform the field regarding responses of patients with varying phenotypic profiles. In addition, another variable requiring further examination in the clinical setting is developmental age. It is clear that developmental stage influences outcomes of enrichment protocols in animal models (Nithianantharajah & Hannan, 2009; Sale, Berardi, & Maffei, 2009). To address this question, testing responses to EE will require that subjects from a large patient cohort are allocated into developmentally relevant age groups to identify critical periods and to track the progression of different subgroups of patients during development.

As discussed here, EE has been found to induce beneficial effects in a range of different animal models of brain disorders. However, the cellular and molecular mechanisms involved in this therapeutic efficacy are yet to be fully elucidated. We can use animal models to further understand mechanisms underlying various neural, cognitive, and behavioral changes in response to EE. Various approaches to these questions show that EE-induced neuroplasticity is complex and can be spatially and temporally modulated. For example, at a cellular level, EE can enhance both adult neurogenesis and synaptic plasticity in the hippocampus of rodents, which may contribute to EE-induced cognitive enhancement (van Praag et al., 2000).

How does EE enhance neural function at molecular and cellular levels? Exploring this key question and associated mechanisms mediating such experience-dependent plasticity not only will be informative on a theoretical level but also will have implications regarding the development of novel therapeutic approaches. The treatment implications of this study potentially include opening avenues for the identification and evaluation of additional drug therapies, in particular, a proposed class of novel therapeutics called "enviromimetics" (Hannan, 2004; McOmish & Hannan, 2007). For this to occur, the molecular mechanisms underlying changes in neural networks and connectivity during EE need to be better understood. Together with standard pharmacotherapies, EE may act in a synergistic manner. Hence, the addition of EE to existing (and future) drug therapies may further improve patient symptoms.

EE in the Treatment of Other Disorders

Woo and Leon (2013) suggest a possible role for noradrenaline, which they state may be associated with changes in brain-derived neurotrophic factor (BDNF) levels in the brain, although no doubt many other molecular systems and signaling pathways may be implicated in the therapeutic effects of EE. Further work is needed to enable accurate real-time measurement of molecular changes in animal models at various stages before, during, and after specific EE paradigms. For patients, the use of peripheral biomarkers and advanced brain imaging technology are required in order to address some of these mechanistic questions.

The findings of Woo and Leon have potential implications not only for ASD but also other disorders. Despite the wealth of preclinical data, there have only been limited attempts to translate animal studies into clinical approaches. For example, the impact of EE on other neurodevelopmental disorders has received little attention and will be worth exploring. In a mouse model of schizophrenia, EE was found to induce significant beneficial effects (McOmish et al., 2008); however, translation of these findings has yet to be systematically attempted in patients. More extensive work has been done involving EE in animal models of neurodegenerative disorders. The first study of EE in any genetic model of a brain disorder was performed in Huntington's disease transgenic mice (van Dellen, Blakemore, Deacon, York, & Hannan, 2000). A follow-up epidemiological study confirmed a role for environmental modifiers clinically (Trembath et al., 2010), although prospective intervention trials are needed. Subsequently, EE has been found to induce beneficial cognitive effects in an animal model of Alzheimer's disease (AD), and neuroprotective effects of cognitive stimulation and physical exercise have been demonstrated for clinical AD (Arendash et al., 2004; Jankowsky et al., 2005; Nithianantharajah & Hannan, 2009). Similarly, EE induces beneficial effects in a neurotoxic model of Parkinson's disease (PD; Faherty, Raviie Shepherd, Herasimtschuk, & Smeyne, 2005), although less EE-relevant clinical work has been done with PD patients.

Closing Comments

Attempts at translating the extensive findings of EE-mediated therapy in animal models remain in their infancy. The study by Woo and Leon (2013) has pioneered EE as a clinical approach for ASD and will no doubt stimulate many follow-up studies. It provides a clear example of how preclinical studies in valid animal models of brain disorders can directly inform clinical investigations and trials. Conversely, clinical discoveries will continue to inform and improve animal models and preclinical studies, so as to facilitate a dynamic cycle of "bench to bedside and back again." Parallel studies in valid animal models and well-characterized clinical cohorts will facilitate the identification of optimal therapies, in which EE-like approaches can be combined with other interventions to improve the lives of the many millions of individuals affected by these disorders.

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